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The Microbiome of Controlled Built Environments

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AFFIDAVIT

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ABSTRACT

The excessive removal of microbes from controlled built environments like intensive care units, operation theaters and especially cleanrooms is daily routine. However, little is known about the effects of this stringent maintenance on co-residential microbiomes in indoor environments. Recent studies have shown that human health relies on the human microbiome, and according to the hygiene hypothesis our well-being depends on the stimulation and influence from surrounding microbiomes of the environment.

Driven by this hypothesis, we investigated effects of microbial confinement and maintenance on the structure and function of microbiomes in different model built environments. In addition, we could reveal the interplay of diverse microbial sources inside the built environment by presenting potted indoor plants as a new driver for the formation of microbiomes indoors and hypothesize potential impacts for human health. Finally, we revealed that embedded and encapsulated bacterial spores could act as a new contamination source (< 0.1 – 2.5 CFU per cm^3) in microbial confined built environments, which needs additional consideration. The application of alternative cultivation strategies, high-throughput-based microarrays (PhyloChip G3) and next generation sequencing technologies (454 pyrosequencing, Illumina MiSeq and HiSeq) helped to identify the gowning area as the main source of microbial dispersal (68%) inside a cleanroom facility. The underrepresented, but highly transferred microbial diversity of intact and potentially viable cells was shifted towards bacterial spore formers (*Ammoniphilus*, *Bacillus*, *Brevibacillus*, *Clostridium*, *Cohnella*, *Desulfosporosinus*, *Geobacillus*, *Paenibacillus*, *Planifilum*, *Sporosarcina*, *Terribacillus*, *Thermoactinomyces*, *Virgibacillus*) and archaea (*Haloferax* and Candidatus *Nitrososphaera*) in controlled built environments. Likewise, functional capabilities of binned genomes changed in the light of microbial confinement in the built environment. Moreover, ornamental plants grown indoors were shown to sustain specific microbiomes in their phyllospheres irrespective of the surrounding microclimates. Therefore, plants represent not only a rich stable source for beneficial microbes (2 – 58%), but could be helpful to control microbial diversity in the built environment using plant-associated biocontrol agents.

This new “bioinformed” knowledge of certain key species and their ecological key functions in controlled and uncontrolled built environments could help to biotechnologically design healthy indoor environments not only on planet Earth, but even beyond.

KURZFASSUNG

Eine intensive Reduktion von Mikroorganismen in kontrollierten Innenräumen wie etwa Intensivstationen, Operationssälen und im Besonderen von Reinräumen ist tägliche Routine. Den möglichen Auswirkungen dieser Reinigungsmaßnahmen auf das uns umgebende Innenraummikrobiom wurde bisher jedoch wenig Beachtung geschenkt. Aktuelle Studien belegen, dass die menschliche Gesundheit auf das Mikrobiom auf und im Menschen angewiesen ist. Darüber hinaus besagt die „hygiene hypothesis“, dass unser Wohlbefinden von der Stimulation und dem Einfluss eines umgebenden Mikrobioms der jeweiligen Umwelt abhängt.

Angetrieben von den Ideen dieser Hypothese wurde die Auswirkung der mikrobiellen Kontrolle auf die Struktur und Funktion des Mikrobioms in verschiedenen Modellumgebungen von Innenräumen untersucht. Zusätzlich konnten wir das Zusammenspiel von Mikroorganismen, die von verschiedenen Quellen aus der Umwelt stammen genauer beleuchten. Ebenso wurden Zimmerpflanzen als eine neue treibende Kraft für die Ausbildung von Mikrobiomen in Innenräumen identifiziert und ihr möglicher Einfluss auf die menschliche Gesundheit diskutiert. Schließlich wurde die Bedeutung von eingebetteten und eingeschlossenen bakteriellen Sporen in mikrobiell kontrollierten Innenraumumgebungen als neue Kontaminationsquelle (< 0.1 – 2.5 CFU per cm^3) aufgezeigt. In einer Reinraumeinrichtung konnte durch die Anwendung alternativer Kultivierungsstrategien, moderner Hochdurchsatzmethoden basierend auf Microarray Technologien (PhyloChip G3) und Next Generation Sequenzierung (454 Pyrosequenzierung, Illumina MiSeq und HiSeq), der Umkleideraum als kritischer Bereich für die Verbreitung (68%) von Mikroorganismen identifiziert werden. Die kontrollierte Umgebung des Reinraumes zeigte zudem eine interessante Veränderung ihrer lebensfähigen mikrobiellen Diversität. Diese lebensfähigen Mikroorganismen, im Besonderen bakterielle Sporenbildner (*Ammoniphilus*, *Bacillus*, *Brevibacillus*, *Clostridium*, *Cohnella*, *Desulfosporosinus*, *Geobacillus*, *Paenibacillus*, *Planifilum*, *Sporosarcina*, *Terribacillus*, *Thermoactinomyces*, *Virgibacillus*) und Archaeen (*Haloferax* and Candidatus *Nitrososphaera*), wurden nicht nur zu einem größeren Anteil in die verschiedenen Bereiche des Reinraumkomplexes übertragen, sondern zeigten gerade in den mikrobiell kontrollierten Umgebungen eine verstärkte Anreicherung. Auf ähnliche Weise veränderte die mikrobielle Kontrolle in verschiedenen Innenraumkategorien auch die

funktionellen Eigenschaften der Mikroorganismen. Darüber hinaus wurde gezeigt, dass Zierpflanzen in Innenräumen das Mikrobiom ihrer Blätter unabhängig von dem sie umgebenden Mikroklima aufrechterhalten können. Somit stellen Zimmerpflanzen nicht nur eine stabile und ergiebige Quelle an nützlichen Mikroorganismen dar (2 – 58%), sondern können mit Hilfe ihrer pflanzenassoziierten Mikroorganismen zu einer biologischen Kontrolle von Innenraumumgebungen beitragen.

Dieses neue „biologisch-informierte“ Wissen über bestimmte Schlüsselspezies und ihre jeweiligen ökologischen Schlüsselfunktionen in kontrollierten sowie in natürlichen unkontrollierten Innenraumumgebungen, könnte in Zukunft dabei helfen, Innenräume zum Wohle der menschlichen Gesundheit biotechnologisch gestalten zu können – und das nicht nur auf der Erde, sondern sogar darüber hinaus.

1. INTRODUCTION

1.1. MICROBIOLOGY OF THE BUILT ENVIRONMENT AND ITS IMPORTANCE FOR HUMAN HEALTH

On planet Earth we are exposed to an incredible number of microbes (10^{29} (Lougheed, 2012)), that even outnumber the amount of stars in the universe (10^{24} according to http://www.esa.int/). Microbes are "metabolic survivalists" and are essential for the ecology of the biosphere. Microbial life is everywhere and there is increasing evidence that we could not survive without it (Gilbert and Neufeld, 2014): Microorganisms provide important nutrients like vitamins in our gut, protect us from diseases (Cho and Blaser, 2012), stimulate our immune system (Vatanen et al., 2016), influence our behavior (Cryan and Dinan, 2012; Diaz Heijtz et al., 2011), support plant growth (Turner et al., 2013), define our diet and well-being (David et al., 2014), detoxify waste and clean our environment (Lovley, 2003). For 4 billion years on Earth, microbial life forms have colonized almost every imaginable environmental niche.

However, the environment is changing as mankind shapes the face of the Earth. Especially since the industrial revolution, a new era – the Anthropocene (Waters et al., 2016) – commenced, and humans not only started unprecedented climate change, but also provided new, so-called built environments, for microbes. Built environments comprise any manufactured product or created structure of mankind on Earth or even beyond in the case of space flight. 1.3 to 6 % of terrestrial area is already covered by residential and commercial buildings, roughly the same amount of land area that is covered by other biomes such as tropical coniferous forests and flooded grasslands (Fig. 1). In addition, the living space of built environments in urban areas often exceeds the covered land area (for instance by 3 times in the case of Manhattan; Fig. 2) (Martin et al., 2015).

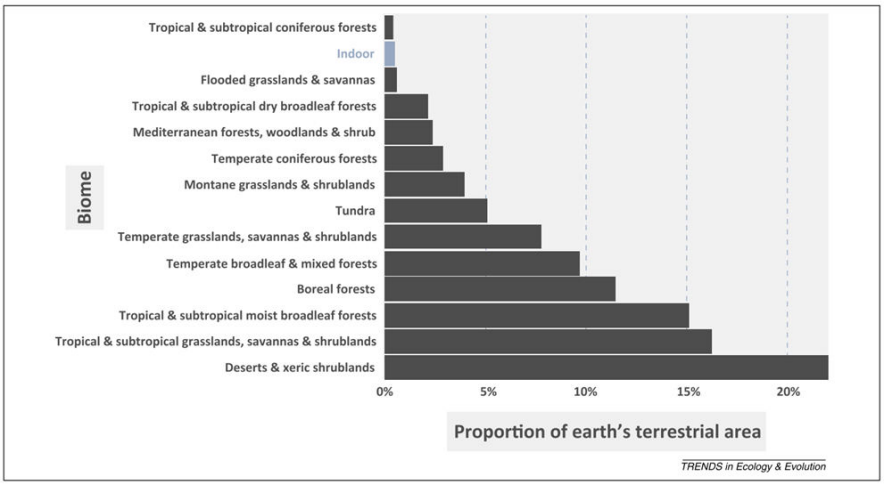

Fig. 1: The relevance of the built environment in the light of covered earth's terrestrial area (Martin et al., 2015)

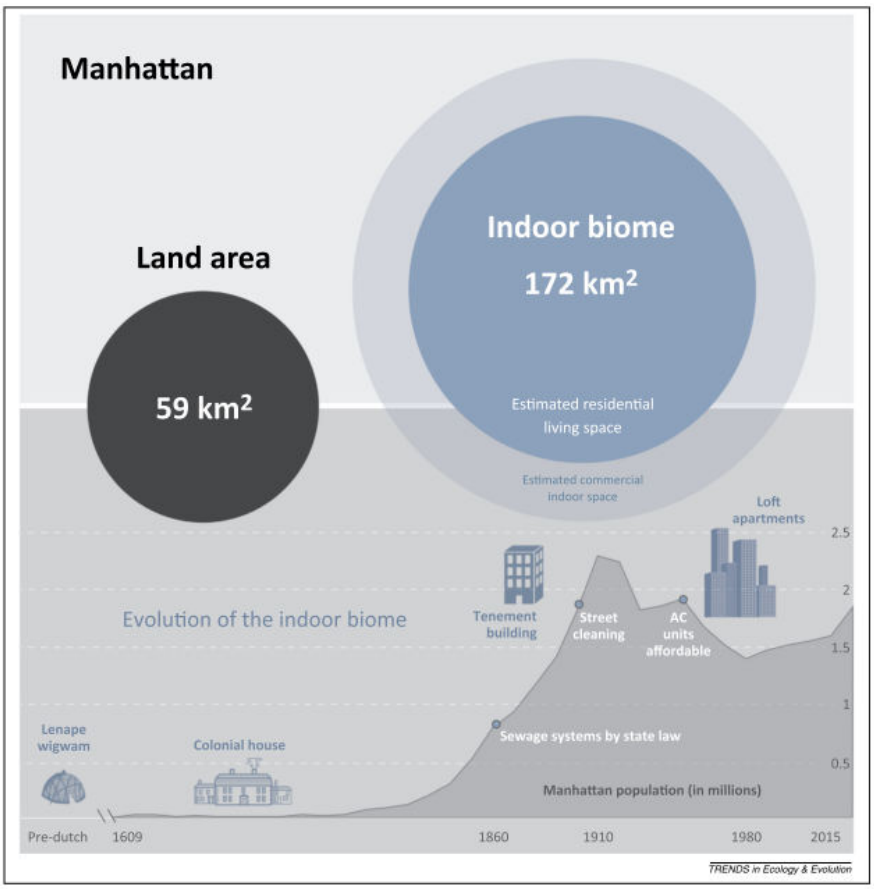

Fig. 2: The trajectory of the indoor biome and its consequences on the covered land area (Martin et al., 2015).

Human beings of industrialized countries spend 90% of their lifetime in these built environments (Kelley and Gilbert, 2013). Most of our daily tasks are conducted in the built environment: we work in offices, sleep in private homes, cure in hospitals, eat, reproduce, and especially share microbes indoors. However, this "encapsulated" lifestyle is not always an advantage. The increasing density of populated urban areas results in challenges: fewer people have regular access to the green outdoors, which results in an increase of diseases associated to human civilization like hypersensitive reactions (allergies and autoimmune disorders) especially in urban populations (Hanski et al., 2012). Moreover, densely populated urban areas and our behavior in a globalized world increases the risks for epidemics and pandemics (Colizza et al., 2006; McManus and Kelley, 2005). The way we control and treat microbes and diseases inside hospitals (Lax and Gilbert, 2015; Arnold, 2014), the architectural design of buildings (Kembel et al., 2012, 2014; Ruiz-Calderon et al., 2016) in the presence of climate change and antibiotic as well as hygienic overuse (Blaser, 2011; Blaser and Falkow, 2009), all together will define human survival and evolution in the built environment. Furthermore, as mankind faces shortage of overall resources our attempts to colonize new worlds beyond planet Earth will be determined by survivability and a healthy life inside enclosed structures of spacecraft, space stations and other confined structures in the presence of microbes.

It is noteworthy that while our health is affected by the microbiome of built environments in the presence of ordinary circumstances it is not usually endangered. However, this condition can change drastically for immunosuppressed patients e.g. in hospital environments where multi-resistant pathogens are on the rise. For a long time, especially molds (beside microbes and insects (Bertone et al., 2016)) were perceived only as contaminants inside our buildings that need to be removed for our and the structure's well-being (Flannigan et al., 2001). Molds for instance might attack the integrity of building structures (Morey, 2011; Pitkäranta, 2011) and are suspected to increase allergic diseases like asthma (Jaakkola et al., 2010; Strachan, 1989; Yamamoto et al., 2011; Karvala et al., 2010; Mutius, 2002; Dannemiller et al.; Riedler et al., 2000; Eder et al., 2006; Reponen et al., 2012) and discomfort syndromes inside buildings (Terr, 2009) beside non-biological causes (Saarloos et al., 2009; Burge, 2004; Codinhoto et al., 2009). Furthermore, obesity (Booth et al., 2005; Papas et al., 2007) and diabetes (Vatanen et al., 2016) could be linked to the built environment, which might imply a connection between physical activity, diet and our microbiome (e.g. by a reduced ratio of Bacteroidetes to Firmicutes in the case of obesity (Cho and Blaser, 2012)).

Bacteria in general are conceived as hazardous life forms inside hospitals, which are responsible for nosocomial infections (Vincent et al., 1995; Plowman, 2000). However, this simple perception neglects the beneficial potential of most bacteria (Oberauner et al., 2013). In addition, despite rigid microbial control, intense use of detergents and antibiotics, our hospital environments or even cleanrooms (see chapter 2.1.) are not invulnerable to microbial contaminants, as a range of different analysis showed in the past (Oberauner et al., 2013; Moissl-Eichinger et al., 2015; Mahnert et al., 2015b). On the other hand, the overall difficulty of establishing germ-free built environments could be an opportunity for reconsidering strict cleanliness in certain areas. Furthermore, an exposure to microbial diversity, especial during first years of infant immune development, has been shown to be important for good health (Eder et al., 2006; Vatanen et al., 2016; Gensollen et al., 2016; Hofer, 2016), as also formulated in the hygiene hypothesis (Rook, 2009; Warner, 2003). Hence, we should design our built environments in a way where we guarantee an exposure to a healthy beneficial microbiome (or probiotics) to direct our immune system into a healthy way and restrict exposures to clean sterile environments to limited and only really necessary applications – such as the application of antibiotics should be limited to urgent cases of bacterial infections as well (O'Neill, 2016).

Clean sterile indoor environments could be even more susceptible to pathogenic outbreaks, since ecosystem diversity is destabilized (Kennedy et al., 2002). Human behavior and maintenance in the built environment might even select for pathogenicity and virulence of fungi (Gostinčar et al., 2011; Zalar et al., 2011) or bacteria. Reducing humidity inside buildings can decrease microbial growth and therefore protect structures from being degraded, but on the contrary also increases infection potential of viruses (Lowen et al., 2007), since dry air reduces the functionality of our nasal mucosa to fend infectious viruses (Arundel et al., 1986; Mäkinen et al., 2009; Williams et al., 1996).

Therefore, positive effects for a building structure's integrity can have adverse effects from a health perspective. If we change our perception of microbes inside the built environment, we will see that we share these habitats with many microbial "co-residents" (Fig. 3 A-C). Especially due to the fact that humans, beside the outdoor air (Meadow et al., 2014; Adams et al., 2015b) and soil particles (Kelley and Gilbert, 2013), are the main source for microbes indoors (Adams et al., 2015a). Recently, ornamental plants have been identified as an additional microbial source (see chapter 2.2.) in built environments (Mahnert et al., 2015a). Beside plants also humans emit millions of microbes indoors (Qian et al., 2012; Meadow et al., 2015; Hospodsky et al., 2012),

which have to adapt further to the abiotic milieu of built environment surfaces and survive in this microbial "wasteland" (Gibbons, 2016). For that reason, passive accumulation of emitted microbes might be the prevalent condition inside buildings in contrast to active succession and proliferation frequently observed in certain outdoor settings.

In general, availability of water inside buildings is crucial for microbial growth. However, not only the level of relative humidity, but much more the humidity directly on surfaces and the time of wetness of a building material, affects survivability of microbes indoors (Zare, 2015; Gibbons et al., 2014). Hence, many studies in the built environment targeted common wet places inside buildings (Angenent et al., 2005; Feazel et al., 2009; Kelley et al., 2004; Miia Pitkäranta, Teija Meklin, Anne Hyvärinen, Aino Nevalainen, Lars Paulin, Petri Auvinen, Ulla Lignell, 2011; Pitkäranta, 2012; Zalar et al., 2011). However, there are also indications that physical and chemical parameters of buildings and their materials are not the main driver for microbial compositions indoors. Much more the interaction of people with a surface could define its microbiome (Lax et al., 2014) e.g. in restrooms (Flores et al., 2011; Gibbons et al., 2014), kitchens (Flores et al., 2013), offices (Hewitt et al., 2012; Chase et al., 2016), or hospitals (Li and Hou, 2003; Oberauner et al., 2013; Hewitt et al., 2013; Brooks et al., 2014), beside location and geography (Chase et al., 2016).

This phenomenon now implies the question if our behavior and maintenance in the built environment not only changes microbial profile structures, but also selects for specific functions, which might affect our health inside buildings (see chapter 2.3.). The next chapter addresses our main research questions in this field in more detail.

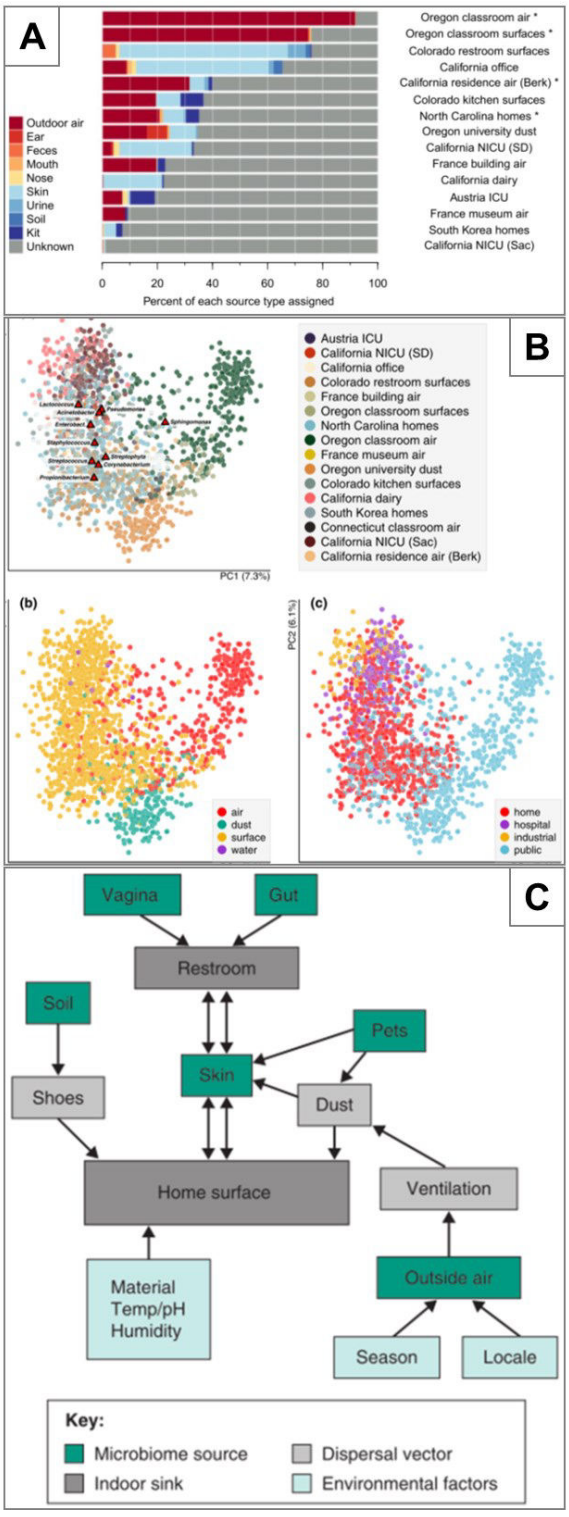

Fig. 3: A) and B) Comparative analysis of some selected studies on the microbiology of the built environment and common microbiome sources (Adams et al., 2015a); C) Microbiome sources, indoor sinks, dispersal vectors and environmental factors in the built environment (Kelley and Gilbert, 2013).

1.2. OBJECTIVE

During the past years we focused on the following main questions in the context of microbiomes in the built environment:

a) How do we alter the viability and structure of microbiomes in the built environment by microbial control and confinement? (see chapter 2.1.)

b) What is the role of plant-associated microbes inside the built environment? (see chapter 2.2.)

c) Which microbiomes (on structural and functional level) are selected in different built environment categories by microbial maintenance and in the light of the microbial interplay from different ecological sources? (see chapter 2.3.)

2. CONCLUSIONS

2.1. MICROBIOLOGY IN CONFINED AND CONTROLLED BUILT ENVIRONMENTS

Confined and controlled built environments like intensive care units, operating theaters, space stations, biosafety labs or cleanrooms are useful model environments to investigate the impact of microbial confinement on the structure of microbiomes inside a built environment.

We focused especially on the environment of cleanrooms for studying microbial maintenance and its consequences on the composition of microbial communities due to the following reasons:

a) Cleanrooms represent well controlled built environments. This controlled setting facilitates scientific interpretations compared to other indoor environments with numerous influencing environmental parameters, which may mask underlying biological effects.
b) Cleanrooms have already been monitored on a microbial level for many years (~1970s) by cultivation dependent and independent methods (partly due to planetary protection regulations (Rummel et al., 2002)). Hence, new data can be analyzed and evaluated much more in the frame of past experiences compared to other built environments.

Since we recently discussed and reviewed these environments in much more detail, (see our review "Microorganisms in confined habitats: Microbial monitoring and control of intensive care units, operating rooms, cleanrooms and the International Space Station" (in peer review) and our book chapter "Complex Indoor Communities: Bacterial Life Under Extreme Conditions in Clean Rooms and Intensive Care Units" (Oberauner et al., 2015)), the following chapter gives only a compact introduction to the confined built environment of cleanrooms.

Cleanrooms are often used in manufacturing plants for numerous industrial processes that require a clean end product, either free of or at least with only low levels of contaminating particles. Dependent on the target of production processes, cleanrooms can be additionally differentiated into particulate-controlled cleanrooms, which are used for instance in the semi-conductor industry and microelectronics, or biocontamination-controlled cleanrooms used for products in the field of aeronautics, the medical and pharmaceutical industry or food technology.

CONCLUSIONS

Since the 1960s, the modern cleanroom has guaranteed clean production by constant air flows and filtering through HEPA (high-efficiency particulate air) or ULPA (ultra-low particulate air) filters (Whitfield, 1964). The extent of cleanliness is indicated by different ISO levels (from ISO 9 to ISO 1; https://www.iso.org/obp/ui/#iso:std:iso:14644:-1:ed-1:v1:en), and is defined by the allowed amount and size of certain particles, beside several other controllable environmental parameters (Whyte, 1999; Gail et al., 2012). To meet these guidelines, materials in cleanrooms are composed of low emitting substances and persons are advised to limit spreading of particles by special gowning equipment and defined behaviors such as slow body movements. For many years, microbial monitoring was routinely based on standardized cultivation dependent methods (Nicholson et al., 2009; Puleo et al., 1977; Powers, 1965; Favero et al., 1966; Vesley et al., 1966; Gen-fu and Xiao-hua, 2007; Whyte and Eaton, 2004b, 2004a; Hussong and Madsen, 2004; Thomas et al., 2005). The application of cultivation independent methods (La Duc et al., 2004, 2009; Moissl et al., 2007; Stieglmeier et al., 2012) as well as alternative cultivation strategies (Stieglmeier et al., 2009; Probst et al., 2010) inside cleanroom environments were mainly propelled by the demands of planetary protection policy (Rummel et al., 2002).

Planetary protection regulations aim to protect extraterrestrial biotopes from Earth-born biological contaminants in order to avoid false positive signals of extraterrestrial life and also to secure the transport of extraterrestrial matter back to Earth for future space missions. Hence, cleanroom isolates were tested extensively for their resistance against numerous environmental stresses (desiccation, radiation, hydrogen peroxide, temperature, heat shock, salinity) to assess their survivability during production processes, space travel, and in suitable extraterrestrial environments (La Duc et al., 2003, 2007).

In compliance with these ambitions, we analyzed the survivability of the spore forming bacterium *Bacillus safensis* isolated from a cleanroom during embedding and encapsulation processes into an adhesive used for spacecraft assembly. Our study revealed for the first time, that bacterial spores retained integrity, germination and cultivation ability and withstand physical stresses during encapsulation and curing processes in a model adhesive. These findings led to the conclusion, that, spacecraft polymers (e.g. adhesive and coatings) are a potential source of microbial contamination (< 0.1 – 2.5 CFU per cm^3), which has to be addressed for planetary protection. Similarly, quality control during food packaging, in the pharmaceutical industry and implant technology should now address these new aspects much more. A useful method for such

a quality control could be the application of physiological fluorescent probes as we presented in our study: “Quantification of encapsulated bioburden in spacecraft polymer materials by cultivation-dependent and molecular methods” (Bauermeister et al., 2014).

All these isolation and characterization efforts in the frame of planetary protection lead to a valuable culture collection of extremotolerant microorganisms from spacecraft assembly cleanrooms for the public (Moissl-Eichinger et al., 2012, 2013; Venkateswaran et al., 2014).

For covering an even broader metabolic spectrum of cleanroom-associated microbes, we applied several alternative cultivation strategies (for details see the study: “Quo vadis? Microbial profiling revealed strong effects of cleanroom maintenance and routes of contamination in indoor environments” (Moissl-Eichinger et al., 2015)) including: anoxic TSA (trypticase soy agar) for anaerobes, R2A (Reasoner´s 2A agar) at pH 10 for alkaliphiles, saline media for halophiles, RAVAN agar for oligotrophes, the *Methanosarcina* medium for archaeal methanogens, beside standard cultivation on TSA and R2A. In addition, we compared all common cultivation independent methods of that time including 16S rRNA gene cloning, microarray technology (PhyloChip G3) and next generation sequencing (454 pyrosequencing). To further reveal the source of cleanroom-associated microbes, we analyzed not only the microbiome of the cleanroom itself, but also the entire cleanroom facility (operated by the Airbus Defence and Space Division, the former EADS company). Our comprehensive study design identified the gowning area as the main source for microbial dispersal (68%) into cleanrooms, and also showed that cleanroom maintenance affected the microbiome structure on all levels. Hence, richness, evenness, diversity, and the total microbial abundance were changed inside controlled cleanroom areas. The human body was identified as an obvious source for microbes (e.g. *Staphylococcus*, *Micrococcus*, *Corynebacterium*, *Propionibacterium*, *Clostridium*, and *Streptococcus*) in all sampled areas. However, each sampled room revealed a distinct microbial profile, which could be linked to: foods in the uncontrolled office room (8%); soils in the gowning room; and unknown sources (55%) for the cleanest areas. Likewise, sequences of common potential pathogens showed higher relative abundances in cleanrooms (18%) and the gowning room (31%) compared to the uncontrolled office environment (13%). Additional analysis using PMA (propidium monoazide) - based molecular assays, allowed the identification of intact and therefore potential viable cells in the overall microbiome structure. Interestingly, only 1-10% of detected 16S rRNA gene

signatures arose from intact cells in the cleanrooms, compared to a proportion of around 45% in uncontrolled areas.

In a subsequent study, we focused on this small fraction of intact and potential viable cells in another spacecraft assembly cleanroom operated by NASA (National Aeronautics and Space Administration); for details see the study: "Cleanroom Maintenance Significantly Reduces Abundance but Not Diversity of Indoor Microbiomes" (Mahnert et al., 2015b). Here, we added ATP (adenosine tri-phosphate) as an additional universal viability marker beside PMA for cultivation independent analysis and targeted the rare cleanroom microbiome by deeper sequencing after PMA treatment of samples. The ATP as well as the PMA-assay, were in accordance to each other and showed a tremendous decrease of the viable microbial abundance and a higher grade of dispersal between the cleanroom and its uncontrolled adjoining facility. Nevertheless, we observed a constant microbial diversity in the presence of this significant reduction of overall microbial abundance. This stable diversity could be explained by a microbial shift in terms of abundance and diversity towards survival specialists like bacterial spore formers (*Ammoniphilus*, *Bacillus*, *Brevibacillus*, *Clostridium*, *Cohnella*, *Desulfosporosinus*, *Geobacillus*, *Paenibacillus*, *Planifilum*, *Sporosarcina*, *Terribacillus*, *Thermoactinomyces*, *Virgibacillus*) and intact archaeal cells (*Haloferax* and Candidatus *Nitrososphaera*) inside the cleanroom compared to its gowning area. Previous studies already indicated the presence of archaea in cleanrooms (Moissl et al., 2008; Moissl-Eichinger, 2011) and other built environments due to their recently described association to humans (Probst et al., 2013). However, this was the first evidence for viable archaea in spacecraft assembly cleanrooms. Finally, we predicted potential functional properties using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) and speculated that despite different microbial profiles of the cleanroom and its uncontrolled adjoining built environment, the functional capabilities of detected microbial communities might be much more similar than expected (see chapter 2.3. for an ongoing analysis).

2.2. THE INTERPLAY OF MICROBIOMES IN THE BUILT ENVIRONMENT

The big impact of the human microbiome on microbial profiles in cleanrooms and other built environments lead to reconsideration of all known microbial sources and the search for new ones in the built environment. The built environment can be perceived as a microbial sink, where humans (Qian et al., 2012; Meadow et al., 2015; Lax et al., 2014), soil, and the outdoor air represent microbial sources (Adams et al., 2015a; Hewitt et al., 2013; Flores et al., 2011, 2013). Microbiomes from different sources will therefore accumulate inside the built environment and this transfer further implies an interplay between microbial communities, which might have previously adapted to different environmental conditions.

Hence, as discussed in previous chapters of this thesis and also shown by many other studies, there is a clear link between humans and the built environment. In addition, there is also a connection between the human microbiome and plant-associated microbiota, since a plant based diet rapidly and reproducibly alters the human gut microbiome (David et al., 2014). However, this dense network circle of microbial interactions was open-ended for a potential link between green plants and the built environment.

Therefore, we tried to close this circle of different microbial networks and hypothesized that potted indoor plants, among others, represent an additional source for the microbiome in the built environment (for details see our opinion article: “Beneficial effects of plant-associated microbes on indoor microbiomes and human health?” (Berg et al., 2014)). Our opinion was impelled by the frequent detection of chloroplast derived as well as soil, root and human-associated archaeal sequences in datasets of the built environment (Oberauner et al., 2013; Probst et al., 2013; Moissl-Eichinger et al., 2015; Mahnert et al., 2015b) and obvious transmission vectors like pollen, seeds (Fürnkranz et al., 2012), plant food products like fruits and vegetables (Flores et al., 2013), beside soil and humans and their pets in general (Dunn et al., 2013; Lax et al., 2014). An overall beneficial effect of green plants onto the built environment was supported not only by psychological effects like the reduction of stress or an increase in creative task performance during the exposure to indoor plants (Fjeld et al., 1998; Shibata and Suzuki, 2004; Chang and Chen, 2005; Bringslimark et al., 2007, 2009; Dijkstra et al., 2008), but much more by the reported capacity of green plants to improve indoor air quality (Orwell et al., 2004) and the

removal of pollutants by their root-associated microbes (Pegas et al., 2012; Kim et al., 2008; Wood et al., 2006).

We could then reveal this beneficial potential of the plant microbiome onto the microbiome of the built environment by a model experiment, where we isolated a common indoor plant (the spider plant *Chlorophytum comosum*) inside a sterile enclosed built environment for half a year (for details see the study: "Microbiome interplay: plants alter microbial abundance and diversity within the built environment" (Mahnert et al., 2015a)). Our study design clearly showed that the plant, significantly shaped the microbial diversity (also confirmed via LEfSe - Linear discriminant analysis of the Effect Size) and abundance (2 – 5 orders of magnitude) for all three domains of life (Archaea, Bacteria and Eukaryota – mainly fungi). Hence, surrounding abiotic surfaces inside the chamber acquired a microbiome, similar to that detected on plant leaves, while the air-born microbiome remained stable over the isolation period. In addition, opposing processes were observed for bacterial (Shannon index H': 5-7) and fungal (Shannon index H': 7-5) diversity on abiotic surfaces of the chamber. This observation might be explainable by a changed microclimate (reduced humidity) during the isolation period, beside a potential but unknown biological effect. The high proportion of intact cells (61%), common beneficial microbes like *Paenibacillus* (Rybakova et al., 2015b; Rybakova, 2015; Rybakova et al., 2015a), and a reduced diversity of fungi with allergic potential e.g. *Aspergillus ochraceus*, *Wallemia muriae* and *Penicillium* spp., are promising indices for future applications of plant-associated bacteria as biocontrol agents inside the built environment or other confined indoor environments like space stations or manned space colonies.

However, remaining uncertainties were in place for the stability of the phyllosphere microbiomes in the presence of different microclimates indoors and the proportion of suitable biocontrol agents on plant leaves for future biotechnological applications to stabilize microbiomes in the built environment. Hence, we investigated a broad range of microclimates and 14 ornamental plants inside the controlled setting of 5 different greenhouses in the botanical garden of Graz (for details see the manuscripts in preparation: "The plant is crucial: specific composition and function of the phyllosphere microbiome of indoor ornamentals" and "Plant-host taxonomy and genotype as drivers of fungal community structure and the antagonistic potential of fungi on the leaves of greenhouse plants"). Furthermore, we targeted potential bacterial and fungal biocontrol agents with TCVA (two-clamp volatile organic compounds assay) (Cernava et al., 2015) against the model plant pathogen *Botrytis cinerea*. Our studies indicate,

that indoor ornamentals sustain their phyllosphere-associated bacterial and fungal microbiome irrespective of the surrounding microclimate in the built environment. In addition, these plants highlight a promising source for bacterial antifungal VOC (volatile organic compound) producers (2 – 58%), which might be applicable to control molds in the built environment with an allergenic potential.

In conclusion, the phyllosphere microbiome is able to stabilize microbial diversity indoors, and may be useful to positively impact human health inside built environments. Our studies indicated that the plant microbiome represents an additional source for beneficial microbes indoors. However, the dependence of plant-associated versus human-associated microbes on overall room maintenance and microbial confinement in the built environment remained obscure. Therefore, a comprehensive study was designed to join our past research efforts and to finally put microbial confinement in the built environment into a larger context.

2.3. MICROBIAL CONFINEMENT OF THE BUILT ENVIRONMENT IN A LARGER CONTEXT

Our prior attempts to evaluate the influence of maintenance and microbial confinement in the built environment were limited to profiles of different microbial taxa in the structure of the microbiome. However, as functional predictions of our datasets already indicated, the extent of functional redundancy might play an important role in the built environment. For that reason, we designed a comprehensive study of diverse built environments differing in the level of microbial confinement and plant versus human-associated microbial sources using shotgun metagenomics supported by 16S rRNA gene amplicon analysis, to assess relations between functional capabilities and microbial profiles in respect of environmental maintenance (for more details see the manuscript in preparation: "The altered microbiome of confined built environments").

To date, detailed functional analysis of microbial-confined built environments are still rather scarce. Again, the cleanroom model served as a first target to reveal functional capabilities in a confined habitat (Weinmaier et al., 2015) and was based on the very same samples as in our study "Cleanroom Maintenance Significantly Reduces Abundance but Not Diversity of Indoor Microbiomes" (Mahnert et al., 2015b).

In this study, more strictly aerobic and non-spore-forming taxa were identified as a discriminative feature for the uncontrolled adjoining facility, while the controlled cleanroom was characterized by facultative and obligate anaerobes and spore-forming taxa. On functional level, metabolic pathways in cleanroom samples showed an increase in nitrogen and vitamin B6 metabolism, as well as ABC transporters and a decrease in pathways associated to peroxisome and folate biosynthesis. The uncontrolled adjoining facility was specified by a reduction of acetyltransferases, methyltransferases, lysine degradation and genetic information processing. These observations contributed to the conclusion that the microbial population of the cleanroom was less dependent on oxygen and more amenable to energy sources like nitrogen. Furthermore, the application of PMA allowed the detection and reconstruction of a low abundant human-associated virus (human cyclovirus 7078A) and a phage of *Propionibacterium* (phage P14.4) from the dataset. Beside viral sequences, eight bacterial lineages (including *Helicobacter*, unclassified Bacilli, and Pleosporaceae) could be positively correlated to human cells. On the contrary, five bacterial taxa (unclassified Bacillales, *Bacillus*, unclassified Clostridia, *Clostridium*

and Propionibacteriaceae) showed negative correlations to human signatures and were therefore suspected to arose from a soil-associated origin (Weinmaier et al., 2015).

Likewise, our comparative analysis of many different built environments (public buildings, public houses, private houses, an intensive care unit, and a cleanroom facility) showed an effective microbial confinement by a clear positive correlation of Eukaryota with more controlled built environments. On the contrary, diversity of the entire biome showed negative correlations with increasing levels of confinement. Distinct bacterial populations were perceived for public buildings and public and private houses (*Nocardioides*, *Arthrobacter, Exiguobacterium sibiricum*, *Microlunatus phosphovorus Knoellia aerolata*, *Janibacter hoylei*, *Kocuria palustris*, *Lactococcus garvieae*, *Weissella confuse*, *Leuconostoc mesenteroides*, *Streptococcus parauberis, Staphylococcus vitulinus, Macrococcus caseolyticus*), which were potentially derived from soil, air, plants, dairy products, fermented foods, fruits and vegetables, the microbial flora of mammals and meat products. Opposed to these detected populations, confined built environments of the intensive care unit and the cleanroom facility showed a high abundance of human-associated bacterial populations (*Propionibacterium acnes, Acinetobacter johnsonii*) including potential opportunistic pathogens (*Staphylococcus aureus, Stenotrophomonas maltophilia, Acinetobacter baumannii*) and only a few beneficials (*Pseudomonas putida*). Some of these taxa (*Pseudomonas, Propionibacterium* and *Porphyromonas*) could be identified as a discriminant feature for controlled built environments (via LEfSe analysis) beside viral sequences of humans (herpes virus and papilloma virus), arthropods (mites) and insects (lices, cockroaches). The diversity of arthropods inside the built environment was just recently characterized in more detail by Bertone and coworkers (Bertone et al., 2016). Distinctive profiles of the biome structure in different sampled categories of the built environment found also consonance into encoded functional capabilities. Hence, uncontrolled built environments were primarily colonized by robust gram-positive bacteria arising from outdoor sources, which had to encode especially for functions to adapt to a varying microclimate (heatshock, fatty acid metabolism), UV radiation (DNA repair systems), and nutrient acquisition (heme and hemin uptake, general processes of carbon metabolism as well as steps involved in glycolysis and gluconeogenesis). Against that, the constant moderate microclimate of controlled built environments selected for gram-negative bacteria and was highly influenced by human-associated bacteria. The close association to a eukaryotic host in this environment resulted in discriminant features for e.g. iron acquisition. However, the greater level of microbial confinement by regular cleaning resulted in functions

associated to oxidative stress together with functions for membrane transport, secretion and apoptosis to gather nutrients from a highly competed oligotrophic environment. Regular attack by cleaning reagents and toxins, were answered by higher capabilities to degrade xenobiotics, geraniol, limonene, pinene, naphthalene, bisphenol, chlorocyclohexane, and chlorobenzene. Finally, microbial confinement and maintenance resulted in broad functional capabilities for drug metabolism and an overall higher level of virulence, disease, defense and resistances also on the level of individual binned genomes of closely related species of the genus *Acinetobacter* obtained from all sampled built environments.

These preliminary observations are summarized in a model for microbial confinement and its effect on structural and functional microbial profiles in the built environment (see Fig. 4).

Our study shows that more natural uncontrolled built environments feature a higher diversity, with complex microbial interactions and functional networks. On the contrary, controlled built environments showed a depleted diversity, an increase in human-associated microbes and particles, with changed functional capabilities. According to that, microbial resistance, virulence and defense might be a key component of microbial communities naturally selected in microbially confined built environments, while natural uncontrolled built environments are colonized by microbes from the outdoor environment, which are adapted to changeable niches and survival in a dynamic environment by a broad and balanced set of functional capabilities.

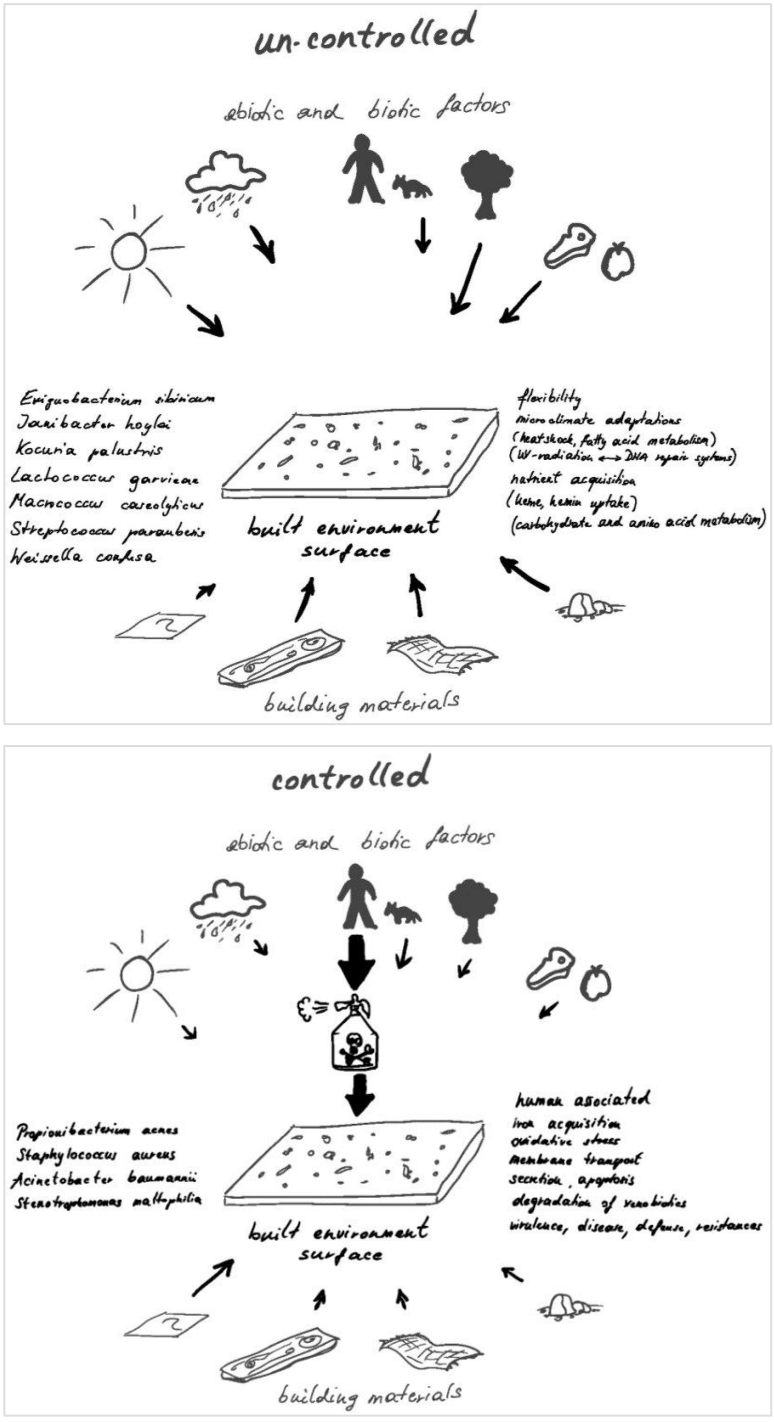

Fig. 1: Suggested model for microbial and functional changes in uncontrolled and controlled built environments. Diverse abiotic and biotic factors as well as building materials on common surfaces in the built environment are illustrated together with detected microbial and functional profiles. The importance of respective environmental factors in each category of the built environment is indicated by length and size of shown arrows.

3. OUTLOOK

Microbial confinement of an indoor environment changes environmental conditions for microbes. Hence, isolation from the outdoor environment increases for instance the relative abundance of shed skin cells and associated human microbiota. A higher proportion of human-associated microbiota in our buildings may result in a higher potential of the surrounding microbial cloud (Meadow et al., 2015) to influence our immune system and affect our health inside buildings either positively or negatively. Our studies indicated that the microbiome structure is already triggered at the level of architecture, microclimate and maintenance inside confined areas. The low exchange with the outdoor environment reduces microbial diversity inside buildings and weakens its overall stability towards the invasion of pathogens (Kennedy et al., 2002). We speculate that regular cleaning might destabilize existing microbiome structures and selects for more problematic germs on a few defined abiotic materials. Diseased occupants might act as vectors to spread germs between different confined habitats. Then, a reduced competition on mainly sterilized surfaces would enable a faster colonization of indoor spaces by microbial germs. Dry air and the reduction of relative humidity in modern buildings could have adverse effects on protective performances of our immune system on mucus layers in the respiratory tract (Lowen et al., 2007; Arundel et al., 1986; Mäkinen et al., 2009; Williams et al., 1996). Constant warm air in buildings could further support the colonization of surfaces by human-associated microbes and increase the survivability of potential pathogens on surfaces. The fact is, humans face increased problems (e.g. nosocomial infections (Lax and Gilbert, 2015)) in microbial-confined built environments, especially immune compromised patients. Hence, we suggest that confinement of a built environment should be limited to defined areas. In all other areas of a built environment, air conditioning systems that provide a constant but inconvenient microclimate and limit the exchange with outdoor air should be reconsidered or modified. Building materials should be diverse to allow a higher microbial diversity. In addition, materials of natural sources are preferred to allow the transfer of already intact and stable microbial communities to a new built environment. Active microbial stimulations (via probiotic cleansers) of surfaces in the built environment are in the early stages of development, but are still questionable for broad applications. Nevertheless, the interruption of human-associated microbial "monocultures" in the built environment could be a beginning. Therefore we suggest the

introduction of green ornamental plants and associated microbiomes from outdoors as an easy way to increase microbial diversity indoors. We hope that the presence of diverse and stable microbiomes in combination with plants inside healthy buildings will result in a higher task performance in offices and a higher air quality.

Research on the microbiology of built environments in consonance with latest findings of host based microbiome research, has potential to reduce likelihoods for diabetes and obesity as well as other diseases of civilization in the built environment, a reduced risk to develop atopic diseases like allergies and asthma for our infants, lower proportions of nosocomial infections in hospitals, safer products and a higher safety for employees, and finally lower costs for maintenance indoors. To achieve this objective, we envision biotechnologically engineered microbiomes supported by a bioinformed design as suggested by (Green, 2014), on structural and functional levels to generate healthier indoor environments not only on planet Earth but even beyond.

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6. ACADEMIC CURRICULUM VITAE

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Personal Information

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6.1. EDUCATION AND CERTIFICATES

1989-2002	School education in Austria (Vienna, Bodensdorf, Villach and Graz); University-entrance diploma at the Bischöflichen Gymnasium Graz (Matura)
2003-2004	12-month civilian service in a facility for mentally handicapped persons
2004-20012	Bachelor and Master studies in molecular microbiology at the Karl-Franzens University and the Graz University of Technology
2011-2012	External master thesis at the Institute for Microbiology and Archaea Center of the University Regensburg (Germany; supervisor: C. Moissl-Eichinger) in cooperation with the Institute of Environmental Biotechnology of the Graz University of Technology in the context of an ERASMUS study –and internship stay.
2012	Graduation with honors at the Karl-Franzens-University and the Graz University of Technology
Since 2012	Employment as a scientific assistant and PhD candidate at the Institute of Environmental Biotechnology of the Graz University of Technology, Graz, Austria

6.2. PROFESSIONAL EXPERIENCE

2008	Research on a (first) archaeal transformation system based on the hyperthermophilic archaeon *Pyrococcus furiosus* at the Institute for Microbiology and Archaea Center of the University Regensburg, Germany
2010	Research on moss-associated archaea at the Institute of Environmental Biotechnology of the Graz University of Technology
2011-2012	Research on encapsulated bioburden (bacterial spores) in spacecraft materials and archaeal methanogens from spacecraft assembly cleanrooms and intensive care units of hospitals at the Institute for Microbiology and Archaea Center of the University Regensburg, Germany
Since 2012	Research on the microbiome interplay between controlled (intensive care units, spacecraft assembly cleanrooms) and uncontrolled (plant-associated) built environments at the Institute of Environmental Biotechnology of the Graz University of Technology

6.3. SELECTED PROFESSIONAL ACTIVITIES

Since 2010	Attendance and funding for talk and poster presentations on several international conferences and workshops in Austria, Germany, USA, Poland, The Netherlands and Italy
2012-	Research stay as a JVSRP (JPL visiting student research program) at the NASA Jet Propulsion Laboratory of the California Institute of Technology (Caltech), Pasadena, CA, USA
2013-2014	Scientific organization of the university course “clean room engineering” at the Graz University of Technology
2013	Organization of the “planetary protection – extremophiles from deep sea across cleanrooms to deep space” symposium at the Graz University of Technology, Austria

6.4. SCIENTIFIC TALKS AND PRESENTATIONS

Alexander **Mahnert**, Christine Moissl-Eichinger, Parag Vaishampayan, Thomas Rattei, Henry Müller, Alexander J. Probst, Lisa Oberauner-Wappis, Roscel Amor Ortega, Kasthuri Venkateswaran, and Gabriele Berg. (2016) Spacecraft assembly cleanrooms exhibit an exceptional microbiome. Invited talk. NASA Jet Propulsion Laboratory (Caltech), Pasadena, CA, USA

Alexander **Mahnert**, Christine Moissl-Eichinger, Parag Vaishampayan, Thomas Rattei, Henry Müller, Alexander J. Probst, Lisa Oberauner-Wappis, Roscel Amor Ortega, Kasthuri Venkateswaran, and Gabriele Berg. (2015) The microbiome in spacecraft assembly cleanrooms and built environments. Astrobiology and Space Medicine Workshop, Graz, Austria

Alexander **Mahnert**, Christine Moissl-Eichinger, Parag Vaishampayan, Thomas Rattei, Henry Müller, Alexander J. Probst, Lisa Oberauner-Wappis, Roscel Amor Ortega, Kasthuri Venkateswaran, and Gabriele Berg. (2015) Microbiomes of the built environment are shaped by our room maintenance. 2nd Theodor Escherich Symposium, Graz, Austria

Alexander **Mahnert**, Christine Moissl-Eichinger, Tobija Glawogger, Henry Müller, Roscel Amor Ortega, Gabriele Berg. (2015); Control of the indoor microbiome by plants? Biodiversity & Biotechnology workshop, Graz, Austria

Alexander **Mahnert**, Christine Moissl-Eichinger, Henry Müller, Alexander J. Probst, Roscel Amor Ortega, Parag Vaishampayan, Kasthuri Venkateswaran, Gabriele Berg. (2015); Indoor Microbiomes change when we try to control them; Healthy Buildings America 2015, Boulder, CO, USA.

Alexander **Mahnert**, Christine Moissl-Eichinger, Henry Müller, Alexander J. Probst, Roscel Amor Ortega, Parag Vaishampayan, Kasthuri Venkateswaran, Gabriele Berg. (2015); We are not alone! – Microbiomes in controlled and uncontrolled built environments; Healthy Buildings Europe 2015, Eindhoven, The Netherlands.

Alexander **Mahnert**, Parag Vaishampayan, Anna Auerbach, Alexander J. Probst, Christine Moissl-Eichinger, Kasthuri Venkateswaran and Gabriele Berg (2013); Viable Microbiome of a Spacecraft Assembly Clean Room – a genetic approach; How dead is dead III: Life cycles conference of the Association for General and Applied Microbiology – (VAAM), Berlin, Germany

Alexander **Mahnert**, Alexander J. Probst and Christine Moissl – Eichinger (2013), Monitoring the Physiological Status of Polymer-embedded *Bacillus safensis* Spores by Fluorescent Dye Staining; How dead is dead III: Life cycles conference of the Association for General and Applied Microbiology – (VAAM), Berlin, Germany

6.5. POSTERS

Alexander **Mahnert**, Rocel Amor Ortega, Henry Müller, Christine Moissl-Eichinger and Gabriele Berg. (2014); Effects of indoor plants on and from their surrounding microbiome in built environments. Microbiology and Infection 2014, 4th Joint Conference of the German Society for Hygiene and Microbiology (DGHM) and the Association for General and Applied Microbiology (VAAM), Dresden, Germany

Alexander **Mahnert**, Matt Christensen, Kasthuri Venkateswaran, Gabriele Berg and Parag Vaishampayan. (2013); Metagenomic Approach to Predict Functional Capabilities of Microbes in Clean Room Facilities; best Poster Presentation Award for the Extremophiles category; 13th European Workshop on Astrobiology of the European Astrobiology Network Association (EANA) in Szczecin, Poland

Alexander **Mahnert**, Parag Vaishampayan, Kasthuri Venkateswaran and Gabriele Berg (2013); Microbiome of a Clean Room and its Associated Environment; 113th General Meeting of the American Society for Microbiology (ASM) in Denver, CO, USA

Alexander **Mahnert**, Lisa Oberauner, Christoph Högenauer, Karl-Heinz Smolle, Alexander Probst, Christine Moissl-Eichinger and Gabriele Berg (2012); Microbial communities of two different extreme indoor environments; 4th American Society for Microbiology (ASM) Conference on Beneficial Microbes in San Antonio, TX, USA

Alexander **Mahnert**, Alexander Probst, Christina Weber, Reinhard Wirth, Klaus Haberer and Christine Moissl-Eichinger (2011); Detection, physiological analysis and quantification of spores encapsulated in modern spacecraft hardware materials; 11th European Workshop on Astrobiology of the European Astrobiology Network Association (EANA) in Cologne, Germany

Alexander Probst, Alexander **Mahnert**, and Christine Moissl-Eichinger (2011). Propidium Monoazide – A Marker for Non-viable Bacterial Endospores in Fluorescence Microscopy. 11th European Workshop on Astrobiology of the European Astrobiology Network Association (EANA) in Cologne, Germany

6.6. AWARDS

2013	Best Poster Presentation Award for the Extremophiles category; 13th European Workshop on Astrobiology of the European Astrobiology Network Association (EANA) in Szczecin, Poland
2015	Best Podium Award at the Healthy Buildings America 2015 conference in Boulder, CO, USA

6.7. EXPERTISE

Molecular microbiology, microbial ecology, bioinformatics, statistics, microscopy, cultivation of extremophiles

6.8. PUBLICATIONS IN THE LAST 5 YEARS

Bauermeister A, **Mahnert A**, Auerbach A, Böker A, Flier N, Weber C, et al. (2014). Quantification of encapsulated bioburden in spacecraft polymer materials by cultivation-dependent and molecular methods. *PLoS One* 9:e94265.

Berg G, **Mahnert A**, Moissl-Eichinger C. (2014). Beneficial effects of plant-associated microbes on indoor microbiomes and human health? Front Microbiol 5:1–5.

Mahnert A. (2012). Vegetative Microbes and Spores Encapsulated in Spacecraft Materials: Molecular Strategies for Determining their Abundance and Physiological Status. Graz University of Technology, Graz, Austria.

Mahnert A, Moissl-Eichinger C, Berg G. (2015). Microbiome interplay: plants alter microbial abundance and diversity within the built environment. Front Microbiol 6:1–11.

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ad chapter 2.1. Microbiology in confined and controlled built environments

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10) **Alexander Mahnert**, Christine Moissl-Eichinger, Markus Zojer, Thomas Rattei and Gabriele Berg. The altered microbiome of confined built environments. In preparation

Microorganisms in confined habitats: Microbial monitoring and control of intensive care units, operating rooms, cleanrooms and the International Space Station

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Abstract:

Indoor environments, where people of the Western world spend most of their time, are characterized by a specific microbial community, the indoor microbiome. Most indoor environments are connected to the natural environment by high ventilation, but some habitats are more confined: intensive care units, operating rooms, cleanrooms or the international space station (ISS) are extraordinary living and working areas for humans, with a limited exchange with the environment. The purposes for confinement are different: a patient has to be protected from infections (intensive care unit, operating room), product quality has to be assured (cleanrooms), or confinement is necessary due to extreme, health-threatening outer conditions as on the ISS. The ISS represents the most secluded man-made habitat, constantly inhabited by humans since November 2000 – and, inevitably, also by microorganisms.

All of these man-made confined habitats need to be microbiologically monitored and controlled. However, the application of microbial cleaning and disinfection measures increases the abundance of survival specialists and multi-resistant strains. Application of a constant selective pressure supports microbes with resistance towards antibiotics or chemical and physical treatments.

In this article, we summarize the available data on the microbiome of the ISS and other confined habitats on ground. By comparing the different operating, maintenance and monitoring procedures as well as microbial communities therein, we emphasize the importance to properly understand the effects of confinement on the microbial diversity, the possible risks represented by some of these microorganisms and by the evolution of (antibiotic) resistances in such environments - and the need to reassess the current hygiene standards.

Introduction:

Nowadays, people spend most of their time indoors (up to 90% in industrialized countries; (Hppe and Martinac, 1998)). In particular, the process of westernization has increased the level of urbanization and created new types of microbiome settings that surround us in our living and work space. The microbiome of a built environment is determined by numerous parameters, such as geographic location, type of usage, architectural design, ventilation and occupancy, but mainly by the living inhabitants (humans, animals, plants), as the major source of microorganisms (Califf et al., 2014; Mahnert et al., 2015a; Meadow et al., 2015). In one example, it was calculated that humans emit up to 3.7×10^7 bacterial and 7.3×10^6 fungal genome copies per person and per hour (Qian et al., 2012).

The human body is a holobiont and thus the home of billions of microbes. Every second of our lives, we interact with microorganisms that support our life and health. This cohabitation has evolved over thousands of years, and is characterized by a balanced interaction of three domains of life (Parfrey et al., 2011; Human Microbiome Project, 2012; Probst et al., 2013; Gaci et al., 2014).

Dysbiosis, the disruption of the human-microbial collaboration, is associated with severe disease patterns, such as inflammatory bowel diseases, obesity or diabetes (Cho and Blaser, 2012). In general, these disruptions are characterized by a substantial reduction of the microbial community diversity. It seems the human system can even irretrievably lose microbes that fulfil evolutionally assigned tasks (Sonnenburg et al., 2016).

In the study by Ruiz-Calderon *et al*., different housing types were analysed with respect to the indoor microbial community, starting with jungle villages to highly urbanized living areas in Manaus (Ruiz-Calderon et al., 2016). Although all of the analysed living areas were well ventilated, the housings of higher urbanization level were characterized by a reduced influence of the outer, natural environmental microbiome. In particular, the portion of human-associated microorganisms was substantially increased. Urbanization was suggested to be one of the factors that drive a reduction in microbiological diversity in living and work areas, with unknown effect on the human microbiome and health.

There are many reasons that necessitate stricter confinement for living and work environments than is typical for most people. For the purposes of this review, we are interested in confined habitats as defined by human-populated environments restricted by a number of parameters. The parameters are a restriction of area and space, and restrictions of physical, chemical and biological exchange with the surrounding, natural environment. Such

confined habitats include areas such as intensive care units and operating rooms, where patients need to be protected from infection; cleanrooms, where the quality of products needs to be assured; and the ISS, which is encapsulated due to life-threatening environmental conditions. A summary of the characteristics of the confined habitats addressed in this review is given in Fig.1.

All these environments require microbiological monitoring, and control, since they harbour their own, possibly adapted, microbial community, which is greatly influenced by the maintenance regime.

In this review, we detail the setting, architecture, and control measures of such environments, which influence the internal microbiome tremendously. We hypothesize that all these environments have parameters in common, which shape, in a similar way, the inhabiting microbial community – with a potential effect on humans living and/or working in these areas.

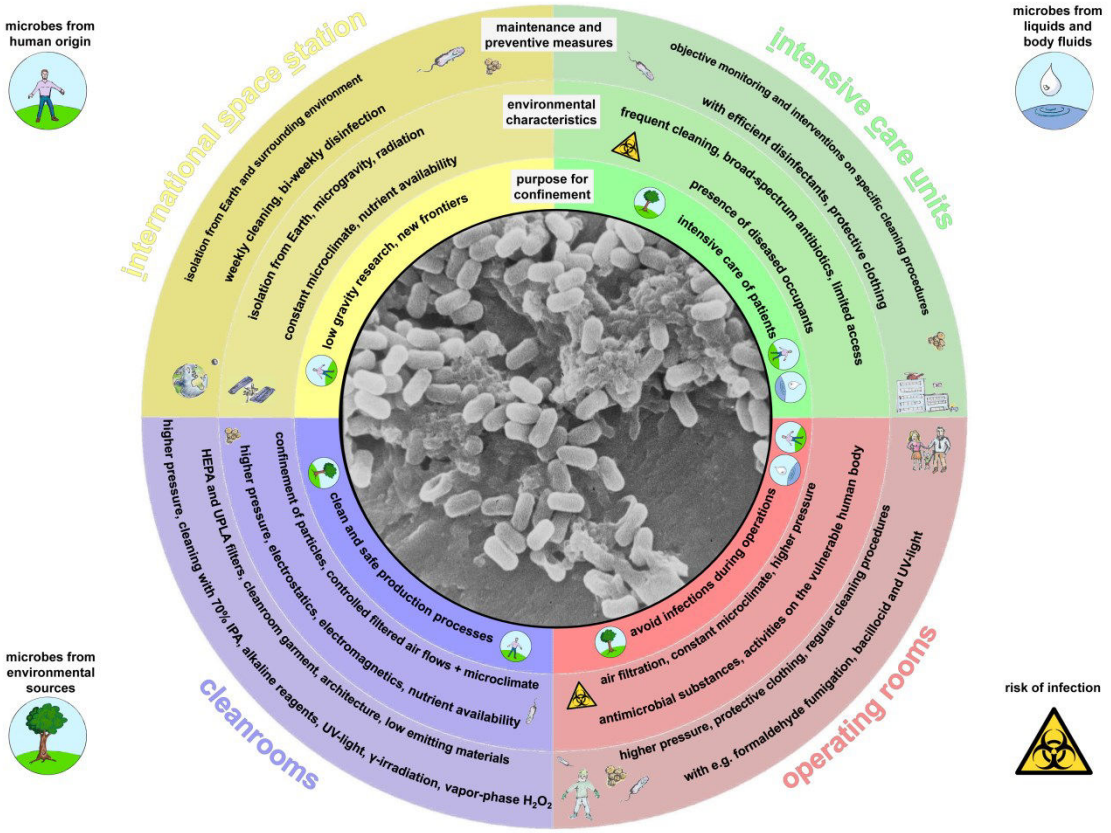

Fig. 1: Graphical display of the confined habitats addressed in this review. Outer rings summarize environmental conditions of the purpose for confinement, some characteristics of each confined environment and overall maintenance and preventive measures in respective built environments. Potential contamination and infection sources are highlighted by small graphics. Inner circle: Bacillus spores, scanning electron micrograph.

The microbiology of intensive care units

Intensive care units (ICUs) are special departments in hospitals that provide intensive medical care for patients suffering from severe and life-threatening diseases or injuries. These units can be divided into several categories, including, for example, neonatal ICUs, pediatric ICUs, psychiatric ICUs, cardiac ICUs, medical ICUs, neurological ICUs, trauma ICUs, and surgical ICUs. Depending on the underlying disease, duration of stay and treatment in ICUs, patients may show higher susceptibility for hospital-acquired infections (HAIs) than healthy individuals due to an overall weak condition, immunosuppression, or disrupted physiological barriers. ICUs are considered potential reservoirs for (opportunistic) pathogenic microbial strains. These microorganisms may thrive on the medical equipment, in other patients, personnel, and the surrounding environment of the hospital (Gastmeier et al., 2007).

HAIs are a serious problem worldwide: in the United States, HAIs are the 6th leading cause of death, killing more people than diabetes or influenza combined (Anderson and Smith, 2005; Klevens et al., 2007), and similar results have been reported from Europe as well (Peleg and Hooper, 2010). For instance, Vincent et al. have estimated the risk for gaining a nosocomial infection in a European ICU to be 45% (Vincent et al., 1995). In general, the risk of acquiring pathogenic infection, in hospital environments is higher than in other environments, and the course of an infection is more often fatal (Centers for Disease and Prevention, 2002; Klevens et al., 2007; Centers for Disease and Prevention, 2010).

Already in the 1980's, specialists in infectious diseases detected that patients in ICUs are infected with nosocomial bacteria considerably more often than patients in other wards in the hospital (Donowitz et al., 1982). Many factors contribute to the increased infection rate in ICUs, including the underlying disease of the patient, the length of the hospitalization, frequency of contact with medical personnel, the number of colonised or infected patients in the same ward, ICU structure (single bed vs. double bed rooms), and the lack of compliance with existing infection prevention guidelines (Siegel et al., 2007). Even the season affects the incidence: in wintertime the risk of acquiring a HAI is smaller compared to other seasons (Schröder et al., 2015). Patient groups that are most often affected are the elderly, premature infants and patients suffering from immunodeficiency (Unahalekhaka, 2011); In the latter, even non-virulent bacteria may cause serious infection and death (Poza et al., 2012).

The risk of infection is increased by invasive, clinically-necessary procedures (like insertion of catheters), but also from architectural properties of the hospital environments (such as ventilation systems (Unahalekhaka, 2011)) or deficient hygiene procedures. For

instance, significantly higher risk for the acquisition of antibiotic resistant microorganisms was observed when newly-arrived patients were placed in rooms that were previously occupied by carriers, despite terminal cleaning of the ICU bed space (Huang et al., 2006; Russotto et al., 2015). This transfer was confirmed by another study, reporting that the infection of the previous room occupant was the most important independent risk factor for infection with *Pseudomonas aeruginosa* and *Acinetobacter baumannii,* two bacteria causing nosocomial infections (Nseir et al., 2011).

The majority of the HAIs is believed to be transmitted directly from patient to patient, but increasing evidence demonstrates that also the medical personnel as well as the clinical environment (i.e. surfaces and equipment) often are a source of infection (Tringe and Hugenholtz, 2008; Caporaso et al., 2012; Passaretti et al., 2013; Salgado et al., 2013). One major vector for cross-contamination are hands of medical personnel, contributing to approx. 20-40% of nosocomial infections (Agodi et al., 2007; Weber et al., 2010). Since infected patients themselves act as a source of microorganisms, frequently touched surfaces close to the patient were heavily contaminated (Wertheim et al., 2005; Pittet et al., 2006). Salgado and co-workers observed that the risk of acquiring a nosocomial infection increased significantly, when the total microbial burden exceeded 500 CFU/100 cm^2 (Salgado et al., 2013).

The link of invasive equipment and the emergence of nosocomial infections has clearly been shown. However, there is also evidence of non-invasive devices to cause ICU outbreaks. Especially, electrical equipment and devices that are difficult to clean (irregular shape, no cleaning regime) have been reported as a source for infection (Russotto et al., 2015).

Hospital textiles are another potential source of HAIs. These textiles are usually reusable and include uniforms, bed linen and pyjamas, as well as privacy curtains and protective clothing of health care personnel. The liberation and dispersal of bioaerosols and fomites from textiles takes place during handling of soiled textiles that have been used by or have been in close contact with an infected patient. It has been shown that antibiotic resistant *Staphylococcus* strains can aerosolize from bed linen during routine handling of bedding and be transmitted via air (Handorean et al., 2015). However, microbial transfer from textiles can be easily prevented by proper laundry procedures (Fijan and Turk, 2012).

Previous studies have shown that opportunistic pathogenic bacteria, such as *Staphylococcus aureus*, various *Enterococcus* species, *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia*, different *Enterobacter* species, *Acinetobacter baumannii* and *Klebsiella oxytoca* are, despite efficient cleaning procedures and disinfectants, commonly found on surfaces such as stethoscopes (Marinella et al., 1997), electronic thermometers

(Livornese et al., 1992), and other equipment routinely used in hospitals (Myers, 1978; Schabrun et al., 2006; Safdar et al., 2012).

Bacteria living in diverse communities at ICUs include pathogenic strains, opportunistic pathogens, as well as harmless and beneficial bacteria. Bacteria found in ICU environments are typically human associated and, due to confinement and strict cleaning procedures, less diverse than indoor environments with unlimited and uncontrolled access. In addition to the above mentioned common hospital pathogens, several genera of opportunistic pathogens have been detected in hospital environments by cultivation and using next generation sequencing methods, including *Actinomyces, Burkholderia, Clostridium, Flavobacterium, Legionella, Neisseria, Propionibacterium, Roseomonas, Streptococcus*, and *Vibrio* (e.g. (Kim et al., 1981; Heeg et al., 1994; Triassi et al., 2006; Hewitt et al., 2013; Oberauner et al., 2013). According to current knowledge, most of the detected bacteria are harmless or beneficial and include, for example, *Bradyrhizobium, Corynebacterium, Delftia, Lactobacillus, Melissococcus, Prevotella, Paracoccus, Sandaracinobacter*, and *Sphingobium* (Hewitt et al., 2013). Bacterial communities in different locations at an ICU vary in species composition and diversity. In general, objects and surfaces near patients, including textiles such as pyjamas, bedlinen, pillows and mattresses, carry more human gut-, hair- and skin-associated bacteria like *Staphylococus, Propionibacteria, Corynebacteria, Lactobacillus, Micrococcus and Streptococcus*, whereas floor and other sites with greater distance to the patient carry more environmental strains. In addition, the abundance of bacteria was higher if samples were taken close to the patient (Handorean et al., 2015; Hu et al., 2015).

(Opportunistic) pathogenic bacteria are typically resistant to various stresses. Due to the extreme selective pressure that confinement and cleaning practices induce, microorganisms living in ICUs develop or acquire resistance mechanisms that allow them to survive in the presence of a vast range of antimicrobial agents used in cleaning and antibiotic treatment, to adapt to extremely low nutrient content, and to persist on dry surfaces for a long time (Poza et al., 2012). In particular biofilms (including multispecies biofilms; (Fux et al., 2005)) can resist common cleaning protocols. Their cells, embedded in the matrix of a biofilm, are considerably more tolerant to desiccation, detergents and disinfectants than planktonic bacteria (Burmølle et al., 2006), making them a highly dangerous infection source for susceptible patients and a critical target for bacterial burden control (Kramer et al., 2006; Hu et al., 2015). Infections caused by biofilm forming bacteria are particularly difficult to treat due to the tolerance of biofilms against host defence mechanisms and antibiotics. In a recent study, Hu and colleagues showed that these diverse biofilms can even tolerate terminal

cleaning procedures of ICU facilities and harbour viable bacteria even after one year (Vickery et al., 2012; Hu et al., 2015). Biofilms have been detected in various locations in ICUs, including a box for sterile supplies, a privacy curtain, a glove box, a noticeboard, and catheters (Perez et al., 2014; Hu et al., 2015). According to Hu and colleagues up to 93% of studied surfaces carried bacterial biofilms (Hu et al., 2015). In addition, the biofilm lifestyle of microorganisms bears a high risk for horizontal gene transfer, consequent spreading of antibiotic resistance and high possibility for recurrence (Fux et al., 2005).

Common examples of multidrug resistance (MDR) are methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) that are also typical components of the ICU microbiome. Often similar cellular mechanisms are used in virulence, antibiotic resistance and resistance to toxic compounds, such as cleaning agents (Daniels and Ramos, 2009; Beceiro et al., 2013).

Cleaning practices at ICUs are an important part of preventing the spread of multidrug resistant organisms, such as MRSA and VRE, which are associated with HAIs, prolonged stays in hospitals, increased mortality rates and higher healthcare costs (Daxboeck et al., 2006).

The cleaning procedures in ICUs are strict, though the practices may vary between hospitals. Depending on the frequency and type of use, dedicated ICU staff and additionally outsourced cleaning personnel are responsible for cleaning hospital interior fittings thoroughly either daily, weekly, monthly or yearly. As one example, the hygiene and cleaning protocol of the Intensive Care unit, Department of Internal Medicine Graz, Medical University of Graz, is shortly mentioned (listed frequencies are minimum demand): E.g. floor is cleaned daily, toilets are cleaned daily (staff toilet) or twice (visitor toilet), shower heads are cleaned once a week, waste is evacuated as necessary and garbage bins are cleaned daily; windowsills, racks, sinks and showers are cleaned daily; laundry is washed daily, vacuum cleaning is done weekly, umbrella holders are cleansed monthly, and telephones and shutters yearly. Exposed surfaces with direct human contact, such as door handles and sinks are cleaned at least daily with cleaning detergents and surface disinfectants. In case of contamination of highly infectious material, including certain viruses and bacteria such as Norovirus and *Clostridium difficile*, a detailed procedure for hand and surface contamination is given: the hands have to be decontaminated with a specific disinfectant detergents under a specific exposure time, depending on which pathogen has caused the epidemic (Cleaning and disinfection protocol, guideline 2000.3116, 7.4.2014. Intensive Care unit, Department of Internal Medicine, Medical University of Graz).

Despite precise protocols and appropriate disinfectants, statistical analyses of data from hospitals has revealed that fatal infections are increasing with more efficient cleaning practices, suggesting that current procedures are inadequate to protect the susceptible patients from serious, life-threatening infections (Arnold, 2014). Efficient cleaning practices are known to decrease, but not eradicate the multidrug resistant organisms living on hospital surfaces (Dancer, 2008). Consequently, new cleaning technologies are being developed. These new methods include for example technologies that are both microbiologically effective and safe to use, such as hydrogen peroxide vapour, and UV light decontamination for terminal cleaning, as well as ultra-microfibers associated with a copper-based biocide. Hydrogen peroxide vapour and UV light can reduce the amount of bacterial cells by at least 4 orders of magnitude, leading to far smaller risks for patients to acquire any multidrug-resistant bacterial infection. These cleaning methods are particularly effective with uneven surfaces and textures that are difficult to access with other methods (Blazejewski et al., 2011). Additionally, bacterial contamination and growth can be reduced by selecting antimicrobial material, such as copper, that can reduce bacterial burden and the possibility for patients to acquire HAI (Schmidt et al., 2015).

Other important factors for preventing infections in ICUs, beside strict cleaning protocols, are monitoring of microbial colonisation and educational interventions of the cleaning procedures and results (Goodman et al., 2008; Carling, 2013). The Centers for Disease Control and Prevention (CDC) published guidelines for monitoring programs for health care workers to improve the environmental hygiene in hospitals, and to provide instant feedback and a possibility to improve the current procedures. These monitoring methods include direct observation of staff performance and protocol compliance, quantitative microbial detection by swab and agar slide cultures, fluorescent markers to identify the frequently touched surfaces, as well as adenosine triphosphate (ATP) bioluminescence for detecting both microbial and non-microbial ATP present in monitored surfaces (Guh and Carling, 2015).

Neonatal intensive-care units (NICUs) are specialized in the treatment of seriously health-threatened or prematurely born infants. In general, infants acquire their microbiome from their mother's vagina (natural birth), skin (caesarean birth) and environment (including the breast milk) emphasizing the role of the NICU's microbiome for the development of a healthy microbiome (Penders et al., 2006; Dominguez-Bello et al., 2010; Brooks et al., 2014). Babies treated in NICUs are often underweight, from low birth weight (<2500g) to extremely low birth weight (<1000g). They have congenital abnormalities, or undergone surgery, and are therefore susceptible to nosocomial infections (Stover et al., 2001; Urrea et al., 2003; Couto et

al., 2007). As in other ICUs, also NICU patients often develop life-threatening infections. Potentially pathogenic bacteria are found in various locations, such as diaper scales, drawer handles, keyboards, sink counters, and door buttons (Hewitt et al., 2013). Epidemiological studies have shown that infective bacteria can spread particularly well via air (Adler et al., 2005), infant incubators (Singh et al., 2005; Touati et al., 2009), sink drains (Bonora et al., 2004), thermometers (Van den Berg et al., 2000), as well as soap dispensers (Buffet-Bataillon et al., 2009) and toys (Naesens et al., 2009). Brooks and colleagues found that tubing, surfaces, incubators, and hands are the most important reservoirs and sources for colonising the premature babies. They also detected that bacteria which later colonize infants' guts can initially be discovered in NICU environmental samples (Brooks et al., 2014). At genus level, typical NICU environmental bacteria include *Staphylococcus, Enterococcus, Acinetobacter, Bacteroides, Burkholderia, Clostridia, Pseudomonas* and *Streptococcus* (Hewitt et al., 2013; Brooks et al., 2014), which are all known to include opportunistic pathogens that potentially are of great risk for immunocompromised patients. However, most of the bacterial genera detected in NICU environments are harmless to humans. If and how these interact with patients and other bacteria is still not understood.

Research has already shown that objective monitoring can significantly reduce the contamination of surfaces near patients, and can point out the weaknesses of current protocols. Monitoring projects have shown that flat surfaces and textiles are easier to keep at the required cleanliness level, whereas more complex surface types, including doorknobs, handles and other irregular surfaces, including electronic equipment are more often cleaned with unsatisfactory quality. Time pressure and lack of adequate instructions may also play a role when the set cleaning standards are not met (Goodman et al., 2008). For example, the 2010 CDC tool kit "Options for Evaluating Environmental Cleaning" offers specific instructions on how to implement monitoring and intervention programs (Carling, 2013). When HAIs are reduced in number via these infection control and prevention programs, also substantial economic benefit can be achieved (Raschka et al., 2013).

Recently, a new and completely different perspective in defeating hospital pathogens has emerged: the interest has shifted from pathogenic bacteria towards the whole microbial communities thriving on different surfaces in hospitals and ICUs, and to a more microbial ecological perspective on how the microbes interact with their environment and other species (Arnold, 2014). It has been shown that a higher microbial diversity can prevent pathogenic infections (van Elsas et al., 2012; Pham and Lawley, 2014), and the idea of supporting the beneficial hospital microbiome by increasing the microbial diversity has raised great interest

(Hewitt et al., 2013; Berg et al., 2015). However, the interaction between pathogenic bacteria, opportunistic strains, and harmful and beneficial microbes in ICUs, as well as in hospitals in general, are not yet understood and more research is still needed.

Operating rooms

Operating rooms (ORs) are important hospital wards where most surgical procedures are performed. These areas are subjected to strict cleaning procedures such as sterilisation, disinfection and removal of contaminants (e.g. dust and organic waste). Cleaning and maintenance schedules are implemented for each operating room according to the surgical procedures performed. All ORs should be cleaned at the beginning of the day, between each surgical procedure, and at the end of the day, followed by a weekly or each second week total clean-up of the entire operating room including walls, floor and ventilation system. In addition, guidelines propose the daily exposure to UV radiation (Rutala et al., 2008; Lives, 2009; Gupta et al., 2015).

ORs are part of operating theatre complexes and these complexes are architecturally divided into four different zones based on the level of cleanliness with the bacterial burden decreasing from the outer to the inner zones. These zones are maintained by a differential decreasing positive pressure to prevent unfiltered air flow towards the inside of the ORs (Spagnolo et al., 2013; Külpmann et al., 2016). The four zones can be divided as follows: a) a protective area that includes the changing rooms for all the medical personnel, administrative staff rooms, pre and post-operative rooms and the sterile and non-sterile stores; b) a clean area that connects the protective area to the aseptic zone; c) the aseptic zone which includes the operating rooms; d) and the disposal area for each operating room (Harsoor and Bhaskar, 2007).

Modern operating rooms are equipped with HVAC (Heating, Ventilation and Air Conditioning) systems to control environmental factors, namely temperature, relative humidity and air flow. The ventilation systems (e.g. with vertical flow, horizontal flow or exponential laminar flow) are equipped with different filters according to the surgical procedures performed. Most ORs have a conventional ventilation system with filters that have an efficiency of 80-95% in removing particles ≥5 μm. In ORs used for orthopaedic and other implant surgeries, the air is filtered through HEPA filters. These filters have an effectiveness of 99.97% in eliminating airborne particles of 0.3 μm size and above (Dharan and Pittet, 2002; Sehulster et al., 2003; Lives, 2009; Spagnolo et al., 2013).

Monitoring the air quality is recommended for each operating room and is often checked by particle count, a method derived from industrial cleanroom standards. This method has been proposed to determine both the effectiveness of the filters in the ventilation system as well as to establish the level of biological contamination (Pasquarella et al., 2000; Gupta et al., 2015).

Many studies have argued that the results of the particle count method do not correlate with the bacterial count results (Landrin et al., 2005; Scaltriti et al., 2007; Cristina et al., 2012). Only two studies have shown that there is a correlation between the number of airborne particles and the number of CFUs. The presence of particles > 5 µm size indicate microbiological contamination in the aerosol (Seal and Clark, 1990; Stocks et al., 2010).

To date, there is no international standard of allowed airborne microbial contamination in operating rooms. Most countries have their own standards: for example, in France the microbiological limits are between 5 and 20 CFU/m^3, which are lower than the limits of the United Kingdom (35 CFU/m^3) and Switzerland (25 CFU/m^3) (Landrin et al., 2005; Cristina et al., 2012). However, facing the increasing use of particle count over microbiological sampling, many countries have established their standards in accordance with the International Standards Organization (ISO) 14644 – Cleanrooms and associated controlled environments (https://www.iso.org/obp/ui/#iso:std:iso:14644:-1:ed-2:v1:en). It is proposed that operating rooms should meet the requirements of a cleanroom of ISO 6 or 7 (explanations see also section on cleanrooms). In contrast, in the ORs equipped with HEPA filters, the levels of an ISO 5 class should be reached (Scaltriti et al., 2007; Chauveaux, 2015).

Active microbial monitoring has been used in most studies as the main method to determine the air cleanliness. This method uses an air sampler to collect a known volume of air which is then blown on agar plates for cultivation-based analyses (Landrin et al., 2005; Cristina et al., 2012).

Besides this method, Friberg et al. (1999) have shown that in operating rooms with laminar air flow the CFU counts on sedimentation plates is a more relevant indicator of bacterial contamination, with CFU levels not exceeding 350 CFU/m^2/h (Friberg et al., 1999). Recently, other methods (e.g. ATP test, fluorescent particle counter) have been implemented to determine the microbiological contamination of the air and surfaces in the operating rooms. Saito et al. (2015) proposed the use of ATP test instead of bacterial culture to identify the contaminated surfaces in operating rooms, while Dai et al. (2015) suggested the use of fluorescent particle counter for real-time measurements of microbes present on aerosol particles (Dai et al., 2015; Saito et al., 2015).

In operating room environments, the presence of microorganisms is closely linked to increased incidence of acquired surgical site infections (SSIs). About 14-20% of all hospital acquired infections are SSIs, leading to an increase in morbidity and mortality, along with rising costs to the healthcare system due to an extended stay in the hospital (Birgand et al., 2015). Most of the microbes causing SSIs have an endogenous source, the patient's microflora. Occasionally, microorganisms acquired from an exogenous source, such as the ORs environment or health care personnel, can be the cause of the development of SSIs (Mangram et al., 1999; Spagnolo et al., 2013).

The factors that may lead to SSIs development are multifarious and can be divided into 3 main categories: (i) patient-related characteristics (e.g. age, obesity, diabetes mellitus and other diseases); (ii) characteristics of surgical procedures (e.g. duration of the operation, type of procedure, surgeon skills, hypothermia control, antibiotic therapy, surgical personnel behaviour and equipment) and (iii) the operating room environment (Mangram et al., 1999; Cristina et al., 2012; Spagnolo et al., 2013).

In most studies, the relation between these factors and the development of surgical site infections has been explored mainly by determining the number of particles in the operating room under different conditions. The number of airborne particles varies during a surgical procedure being higher at the beginning due to patient installation and surgical bed preparation, and an increased movement of the medical personnel (Knobben et al., 2006).

Additionally, the surgical personnel and patients release skin particles (especially when the skin is dry), respiratory aerosols, dust particles and textile fibres containing viable microorganisms in the operating room environment, therefore increasing the overall count of airborne particles (Dineen and Drusin, 1973; Mangram et al., 1999). Moreover, Cristina et al. have shown that the use of certain instruments (e.g. ultrasonic scalpel, laser tissue coagulation), which produce surgical smoke, increases the number of particulates in the OR air during surgical procedures, but the increasing number of particulates was not correlated with the microbial load (Cristina et al., 2012).

Besides the presence of surgical personnel, their behaviour can also lead to an increased number of microbiological particles. Several studies have shown that the number of persons present during a surgical procedure influences the number of airborne particles to a big extent, their movement leads to resuspension of any dust particle settled and the door opening rates cause an increase in the number of bacteria that can enter the ORs (Scaltriti et al., 2007; Lynch et al., 2009; Wan et al., 2011). To lower the particles shed by the health care personnel and to decrease the incidence of SSIs, different guidelines suggest the use of alcohol-based

hand rubs, double gloves, face masks, hoods for covering the hair as well as the use of disposable impermeable garments made of non-woven particles during surgical procedures (Sehulster et al., 2003; Howard and Hanssen, 2007; Humphreys, 2009; Lives, 2009; Salassa and Swiontkowski, 2014). In some studies, the incidence of surgical site infections increased when the health care personnel wore the suits and shoes or used mobile devices both in and out of the operating rooms (Amirfeyz et al., 2007; Hee et al., 2014; Venkatesan et al., 2015).

Up to 30% of all surgical site infections are known to be caused by *Staphylococcus aureus*, especially the methicillin-resistant strains (Anderson et al., 2007). *S. aureus* is one of the most commonly isolated microorganisms from the ORs environment and a typical skin-associated microbe, indicating that ORs are dominated by human associated microbiota (Shin et al., 2015).

In two different studies the number of *S. aureus* has been investigated in different zones of the operating rooms. The number was increased in the critical zone (in close proximity of the patient) in comparison with the intermediate and peripheral zone (Edmiston et al., 2005; Genet et al., 2011).

Besides *Staphylococcus* ssp., other microorganisms have been isolated from operating rooms such as: *Enterobacter* spp., *Micrococcus* spp., *Acinetobacter* spp., *Brevibacterium* spp., *Pseudomonas* spp., *Klebsiella* spp., *Bacillus* spp. and *Escherichia coli* (Edmiston et al., 2005; Wan et al., 2011; Al Laham, 2012; Venkatesan et al., 2015; Verde et al., 2015).

Commonly, the microbiota associated with surgical site infections (SSIs) are investigated by culture-dependent methods and include well known opportunistic pathogens (e.g. *S. aureus*, *Enterococcuss* spp., *Pseudomonas* spp. and *Escherichia coli*). However, a study performed by Wolcott et al. (2009) shows that the vast majority of the microorganisms linked to SSIs is unidentifiable using standard culture methods (Wolcott et al., 2009) and consists mostly of anaerobes (the majority belonging to the genus *Bacteroides*).

Knowing that only a small fraction (around 1%) of the microbial diversity can be cultured and described (Amann et al., 1995), the usage of molecular methods arises as a prerequisite not only for identifying the microorganisms present in the ORs environment, but also for uncovering the mechanisms of their dispersal and exploring the sources of microbiological contamination.

To date, only one study has explored the entire microbiome of an OR by using molecular techniques (Shin et al., 2015). Shin et al. (2015) performed next generation sequencing of the microbial communities present in three OR environments (found in two different hospitals), and proved that the operating room dust contained a microbial community similar to the one

found on human skin (dominated by *Staphylococcus* and *Corynebacterium*). Moreover, *Staphylococcus* strains have been isolated from the dust present on ORs mobile surgery lamps, pointing out a high infection risk associated with the formation of microbial plumes. Overall, the study showed that the microbial communities present in all three operating rooms were similar, and that the bacteria present belonged to the phyla Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria and Cyanobacteria (Shin et al., 2015).

More studies on the microbiome of the operating room environment are needed to identify the main sources of microbial contamination, to understand how these microbes thrive in these controlled environments and how they are transmitted from humans to surfaces and *vice versa*. This would help to optimize stringent maintenance and cleaning procedures and to lower the microbial burden. Furthermore, health care personnel should be instructed on how to perform safer surgeries and how to minimize the microbial shedding during surgical procedures. The recommendations of WHO and CDC guidelines (Sehulster et al., 2003; Lives, 2009) should be applied in each operating room to prevent surgical site infections and avoid unwanted expanse for both the patient and health care facilities.

Cleanrooms

Cleanrooms are facilities used for ensuring quality and safety of many production processes. They are either mainly particulate-controlled (e.g. microelectronics, semi-conductor industry), or additionally biocontamination-controlled in case of food technology, pharmaceutical industry, medical processes (e.g. biosafety labs), aeronautics and many other application areas.

The idea to use a biocontamination-controlled, clean environment to increase hygiene standards was first implemented by the two physicians Semmelweis and Lister in the 19th century. They realized the presence of an "invisible threat", which we nowadays have identified as the presence of (opportunistic) pathogenic microorganisms or viruses. By their developed countermeasures they were able to significantly decrease mortality rates in hospitals (Semmelweis, 1988). However it was Willis Whitfield who created the basis of the modern cleanroom in 1960 and solved the problem of contaminating particles and unpredictable airflows by the application of a constant highly filtered air flow to flush out air impurities (Whitfield, 1964).

A "clean" production process results in a product, which is free of contaminants of concern. Such contaminants can be microorganisms themselves and their remnants,

biomolecules in general, as well as any (inorganic) particulate matter that could affect the production process and the quality of the end product. Nowadays any outdoor air entering the cleanroom is filtered and air inside the facility is constantly recirculated through HEPA (high-efficiency particulate air) and/or ULPA (ultra-low particulate air) filters to prevent contaminants to enter the cleanroom or settle on its surfaces. In addition, most cleanrooms are operated at higher pressures than their outside environment to prevent inadvertent airflows into cleaner areas.

The installation of a clean production line requires proper planning prior to the operation itself, including consideration of specific requirements of the product (Whyte, 2001). Specific decisions have to be taken with respect to operation (i.e. exchange of materials (products) and personnel), maintenance and monitoring (i.e. measurements of air conditions, particles, flow dynamics, acoustics, electrostatics, electromagnetics, contaminating sources, risk and hazard assessments, concepts of air flow facilities, laminar flow cabinets, filter fan units), calculations of energy and media consumptions, as well as hygiene protocols and evaluations (i.e. disinfection, decontamination).

A cleanroom class is defined by its amount of particles of a certain size according to the ISO classification criteria (see also above). Hence, a cleanroom of ISO Class 6 is for instance allowed to contain 10^6 particles equal to and larger than 0.1 μm in size per m^3 of air. This number is then decreasing by 1 log per ISO category resulting in 10^5 for ISO 5, 10^4 (ISO 4), 10^3 (ISO 3), 10^2 (ISO2), and 10 particles for ISO 1, which represents the cleanest level. In case even higher cleanliness is required, so-called insulators can be installed inside a cleanroom environment. Cleanrooms of ISO classification 7 to 8 represent the most common and appropriate levels of cleanliness for many different production lines. Here, classification is based on 0.5 μm- sized and larger particles with limits at 3.5×10^5 for ISO 7 and 3.5×10^6 for ISO 8 per m^3 air, whereas ISO Class 9 (3.5×10^7 particles) corresponds already to the particle concentration observed in uncontrolled areas. Besides the presence of particles, cleanrooms are controlled with respect to temperature and humidity (HVAC systems; heating, ventilating and air conditioning), the kind and quality of gaseous substances, the light source, electrostatics and electromagnetics (Whyte, 1999; Hortig, 2002).

Cleanrooms are often arranged in a sequential manner to guarantee desired conditions on each level. For this purpose, cleaner areas are only accessible after passing other cleanrooms of higher ISO classes in decreasing manner. Passages between different ISO classes and into cleanrooms are often sealed by airlocks or sluice systems, which sometimes include additional air showers and tacky mats. These systems intend to remove dust, soil, skin flakes and many

other contaminating particles associated with a person or item. Work processes, as well as people behaviour and interaction with respective products are strictly predefined to avoid needless spreading of particles. Hence, people in general are advised to perform their duties with slow body movements inside a cleanroom environment. In addition, the staff is equipped with special cleanroom garment that has to be donned in a specific area in a pre-defined order and often includes an overall, pants, bonnet, moustache cover, glasses, gloves, shoe covers, boots and hoods. Previous studies have shown that dispersion rates of microbe carrying particles (MCPs; ≥ 0.5 µm) were substantially reduced from 2.1×10^6 to 1×10^6 per minute, when staff wore cleanroom garment compared to normal indoor clothing (Whyte and Hejab, 2007), emphasizing the effectiveness of such control measures.

Since cleanrooms can harbour entire production lines, these rooms are modular and scalable up to enormous sizes. Depending on the mode of use, cleanrooms can be equipped with diverse machines and furniture. Regardless of its special requirements, installed devices have in common that they should generate minimal air contaminations and are easy to clean. Hence, materials from natural fibers are often excluded from devices used in cleanrooms.

Microbial decontamination actions are performed regularly but without leaving any residues behind. Standard cleaning reagents include alcohols (e.g. 70% (v/v) isopropanol), hydrogen peroxide (e.g. Klercide-CR) and alkaline cleaning reagents (e.g. Kleenol 30 or Jaminal Plus), and could be supplemented with e.g. UV light, γ - irradiation and vapour-phase H_2O_2 treatments. Cleaning schedules can be rather elaborative including extensive repetitions of vacuuming and mopping as well as other cleaning protocols. As a result, microbial abundance is often intensively reduced compared to uncontrolled adjoining facilities. However, harsh environmental conditions and selective pressures in the cleanrooms also result in a microbial shift towards survival specialists like bacterial spore formers or archaea (Mahnert et al., 2015).

Microbial monitoring in biocontamination-controlled cleanrooms is often executed according to standard, cultivation dependent approaches based on the usage of contact plates (nutrient agar plates), witness plates (if specific surfaces are too sensitive to be sampled) or air sampling directly onto nutrient agar plates.

Besides pharmaceutical cleanrooms, also industrial cleanrooms are sometimes required to operate under biocontamination control. Examples are spacecraft assembly cleanrooms that house mission vehicles, intended to land on extraterrestrial areas of elevated risk for contamination with Earth-borne microbes. Such missions are subject to strict planetary protection regulations (Kminek and Rummel, 2015).

First studies that examined the microbial contamination of such industrial cleanrooms were conducted in the 1960s (Nicholson et al., 2009), especially in preparation for the Viking mission to planet Mars (Puleo et al., 1977), starting with the microbial characterization of laminar flow cleanrooms (Powers, 1965). A first report on a comprehensive analysis of a horizontal laminar flow, three conventional industrial cleanrooms, and three open factory areas for the presence of microbial contaminants using witness plates was published by Favero and coworkers in 1966. It was found that the number of CFUs was reduced along with the reduction of particles in samples from the air and surfaces and reached a plateau after several weeks of exposure. Microbial contaminations (mainly vegetative microorganisms of human origin like *Staphylococcus*, *Micrococcus*, *Corynebacterium*, *Brevibacterium*) could be clearly associated with the density and activity of personnel in the cleanroom (Favero et al., 1966). In the 60's, general microbial levels on flat surfaces were evaluated using Rodac (Replicate Organism Detection and Counting) plates. These plates contained Trypticase Soy Agar (TSA) and were, after sampling, incubated at 32°C for 43 h (Vesley et al., 1966). Similar procedures are still used today. Later on, industrial cleanrooms were brought into a broader perspective after comparing their microbial contamination type and levels with those found in hospital operating rooms. The hospital environment harboured at least 1 log higher microbial abundances (based on colony forming units) than the investigated cleanrooms (Favero et al., 1968).

In the case of bioaerosol characteristics, Li and Hou observed only weak relationships among different cleanroom class levels in hospitals and air particle concentrations (Li and Hou, 2003). The index of microbial air contamination (IMA) was proposed as a reliable tool for monitoring surface contamination by settling of microbes from the air and was tested in environments like hospitals, food industries, art galleries, aboard the MIR space station and in open air (Pasquarella et al., 2000).

Several authors discussed the effectivity of microbiological methods and analytical tools to assess the risk of typical microbial contaminants, such as *Staphylococcus*, *Microbacterium* and *Bacillus* (Wu and Liu, 2007) during pharmaceutical production (Whyte and Eaton, 2004a; b) or in aseptic processing cleanrooms (Hussong and Madsen, 2004). Thomas et al. concluded that the aseptic techniques applied by the personnel were more critical in avoiding contamination, than the general level of cleanliness of the environment (e.g. a cleanroom) for compounding drugs (Thomas et al., 2005).

Nevertheless, besides modelling the spreading of contaminants, risk assessments, improving sampling strategies from air and surfaces in various cleanroom settings, most

studies that tried to expand applied methods beyond routine microbial monitoring were conducted in spacecraft assembly cleanroom settings due to planetary protection requirements (Nicholson et al., 2009; Kminek and Rummel, 2015). For planetary protection purposes, the profound knowledge and understanding of the cleanroom and spacecraft associated microorganisms is an important prerequisite for mission success. Besides standard assays based on cultivation of aerobic mesophilic and heat-shock resistant microorganisms, more sophisticated methods have been established. These included for instance the cultivation of microbial contaminants on anoxic TSA, resulting in a collection of more than 100 strains of facultative (*Cellulomonas, Paenibacillus, Staphylococcus, Arsenicicoccus, Dermabacter, Pseudomonas, Stenotrophomonas, Corynebacterium, Enterococcus*) and obligate anaerobes like *Clostridium* and *Propionibacterium* (Stieglmeier et al., 2009; Probst et al., 2010). Isolated bacteria from several spacecraft assembly cleanrooms were extensively tested for their resistance against numerous environmental stresses like desiccation, UV-C irradiation, γ-radiation, 5% (v/v) hydrogen peroxide, temperature extremes from 4 to 65°C up to a heat shock of 80°C, pH 3 and 11, and hypersalinity of 25% NaCl (w/v), in order to understand their potential capacity to survive space flight or under extraterrestrial conditions. Besides extremotolerant *Alphaproteobacteria*, *Betaproteobacteria, Gammaproteobacteria* (*Acinetobacter radioresistens*), *Actinobacteria* and fungi (*Aureobasidium*), highly tolerant spore forming isolates were found, including numerous bacilli, *Geobacillus* (thermophilic), *Paenibacillus* (obligate anaerobes), and other species that revealed halotolerant and alkalo-tolerant characteristics (La Duc et al., 2003a; La Duc et al., 2007).

The application of diverse cultivation strategies and regular monitoring and isolation of microbes from spacecraft assembly cleanrooms resulted in a rich culture collection of extremotolerant microorganisms from confined built environments that is now open to the scientific community at the German Collection of Microorganisms and Cell Cultures DSMZ (Moissl-Eichinger et al., 2012; Moissl-Eichinger et al., 2013) or through the U.S. Department of Agriculture's Agricultural Research Service Culture Collection (Venkateswaran et al., 2014b).

However, beside cultivation based methods, several studies conducted in spacecraft assembly cleanrooms included also (molecular) cultivation independent assays to target microbial diversity and abundance in NASA (National Aeronautics and Space Administration) and ESA (European Space Agency) affiliated spacecraft assembly cleanrooms. La Duc and coworkers used molecular methods in 2003 in addition to culture-based methods to characterize microbial diversity of a cleanroom encapsulation facility and

the collocated Mars Odyssey spacecraft. Predominant species in clone libraries included *Variovorax*, *Ralstonia* and *Aquaspirillum*. The application of various biomarkers such as ATP, LPS (lipopolysaccharides), and DNA to assess contamination of spacecraft and associated environments were reviewed by La Duc *et al.* including even samples from the International Space Station (La Duc et al., 2004). In 2009 DNA microarrays (PhyloChip) were added and compared in-depth to standard cloning methods in a study covering cleanrooms before and after spacecraft assembly at Lockheed Martin Aeronautics Multiple Testing Facility (LMA-MTF), Kennedy Space Center Payload Hazard and Servicing Facility (KSC-PHSF), and the Jet Propulsion Laboratory Spacecraft Assembly Facility (JPL-SAF) (La Duc et al., 2009). Three geographically distinct spacecraft-associated cleanrooms (Jet Propulsion Laboratory, Kennedy Space Flight Center, Johnson Space Center), including air samples, were analysed in another study to determine if microbial populations are influenced by the surrounding environment or cleanroom maintenance. Only a small subset of microorganisms (e.g. *Acinetobacter*, *Deinococcus*, *Methylobacterium*, *Sphingomonas*, *Staphylococcus* and *Streptococcus*) was common to all locations, whereas samples from Johnson Space Center featured the greatest diversity of bacteria, Kennedy Space Flight Center samples were characterized by a high presence of *Proteobacteria* and areas in the Jet Propulsion Laboratory assembly facility harboured mainly *Firmicutes*. The air of these spacecraft assembly facilities contained for instance *Massilia timonae*, *Agrobacterium tumefaciens* and *A. sanguineum*, *Janthinobacterium lividum*, *Wautersia metallidurans*, *Acidovorax temperans*, *Deinococcus geothermalis*, *Delftia acidovorans*, *Gemmata obscuriglobus* and *Methylobacterium fujisawaense* (Moissl et al., 2007). In addition to NASA operated spacecraft assembly cleanrooms, their European counterparts used by ESA were investigated for their microbial abundance and diversity as well (Stieglmeier et al., 2012). However, not only Bacteria could be associated to human-controlled environments but also signatures of Archaea (*Thaumarchaeota,* closely related to *Nitrososphaera gargensis*; and *Euryarchaeota* like halophilc and alkaliphilic *Halalkalicoccus*, and the methanogen *Methanosarcina*) were detected by molecular methods and could be visualized by FISH (fluorescence *in situ* hybridization) (Moissl et al., 2008; Moissl-Eichinger, 2011).

Similarly like Bacteria, Archaea seem to be transferred by humans into cleanroom environments (Probst et al., 2013). Although they were found to be less (3 logs) abundant than bacteria (2.2×10^4 archaeal cells per m^2 cleanroom surface determined via quantitative PCR), they seem to be a constant microbial contaminant. Recently, an shotgun metagenomic approach using multiple displacement amplification (MDA) completed the picture of

microbial life in a cleanroom by the detection of Eukaryotes (*Acanthamoeba* and fungi e.g. *Leotiomyceta*, *Exophiala*, *Mycosphaerella*) and diverse viruses (Weinmaier et al., 2015).

New molecular methods like next generation sequencing nowadays allow not only a much better assessment of the total microbiome inside confined habitats like cleanrooms, but can additionally be enriched by different assays to target potential viable microbial communities. For instance the application of propidium monoazide (PMA), a chemical compound that masks DNA of dead cells from further downstream molecular analysis, revealed a remarkable proportion of dead cells (up to 99%) compared to other uncontrolled built environments (Vaishampayan et al., 2013; Mahnert et al., 2015b). The viable portion of the cleanroom environment included bacterial spore formers, such as *Ammoniphilus, Bacillus, Brevibacillus, Clostridium, Cohnella, Desulfosporosinus, Geobacillus, Paenibacillus, Planifilum, Sporosarcina, Terribacillus, Thermoactinomyces, Virgibacillus*) and archaea (*Haloferax* and Candidatus *Nitrososphaera*) (Vaishampayan et al., 2013; Mahnert et al., 2015b).

Moreover, viability assays using PMA were shown to increase the traceability of low abundant taxa of the rare viable biosphere (Mahnert et al., 2015b) and help to assess the entire complexity of microbiomes in confined environments which are dominated by DNA signatures of dead cells (Weinmaier et al., 2015). Hence, the importance to include differentiated methods targeting the total microbiome and that of viable or intact cells is of particular relevance in microbially controlled low biomass environments, to allow a less biased picture of the microbial DNA-based inventory.

The investigation of a whole cleanroom facility including adjoining facilities besides actual controlled cleanrooms highlighted the critical role of the gowning area. These areas are located in front of restricted clean zones, and were identified as the major location and source of microbial contaminant dispersal into cleanrooms (Moissl-Eichinger et al., 2015). Moreover, the authors of this study applied a broad spectrum of methods and compared standard cultivation techniques (TSA, R2A), adapted cultivation protocols for anaerobes (anoxic TSA), alkaliphiles (R2A at pH 10), halophiles, oligotrophes (RAVAN agar), methanogens (*Methanosarcina* medium) and various (molecular) cultivation-independent methods including 16S rRNA gene cloning, micro-array technology (PhyloChip) and next generation sequencing (454-pyrosequencing). Interestingly, against expectations, high throughput next generation sequencing technologies could not cover all cultivated microbes (Moissl-Eichinger et al., 2015). However, due to targeting 16S rRNA genes, this study missed the entire microbial complexity as accessible through broader or even untargeted approaches (Vaishampayan et al., 2013; Mahnert et al., 2015b). Hence all methods, even state-of-the-art,

have their individual advantages, disadvantages and limitations. However, in combination they have the potential to lead to a more complete picture of microbes inside the extreme environment of the cleanroom (Moissl-Eichinger et al., 2015).

In conclusion, from a microbial perspective, a cleanroom is an extreme environment, where strict maintenance and overall lack of nutrients complicate microbial growth. The human body serves as a continuous source of microbial contaminants, although also environmental sources (such as soil, dust particles and aerosol droplets) represent another common source of cleanroom microbes. Once transferred to the cleanroom environment, microbes adapt their metabolism (Weinmaier et al., 2015) to withstand harsh conditions, responding to starvation, by reduction of overall metabolic activity (dormancy) and spore formation. Hence, cleanroom maintenance selects especially for microbial adaptation and survival specialists – and thus enriches microbes posing a higher risk for planetary protection. For those purposes, cleanroom maintenance and the design of its infrastructure should be reconsidered and the necessity as well as impracticality of overall sterility in a cleanroom should be critically discussed in the future.

ISS & human longterm Space travel (Mars & beyond)

Another confined man-made habitat exists about 400 km above ground: The International Space Station (ISS), one of the biggest and most complex international scientific projects in history, is circling our planet in low Earth orbit. As joint venture of the five space agencies of USA (NASA), Europe (ESA), Russia (Roscosmos; Russian Federal Space Agency), Canada (CSA; Canadian Space Agency), and Japan (JAXA; Japanese Aerospace Exploration Agency), the ISS is organized in modules. The first module, namely the Russian Zarya module, was launched in 1998 and since 30th October 2000, the ISS has been constantly inhabited by humans. While the ISS kept growing by the addition of new modules over the years, also the crew size increased from initially three crew members to six international astronauts and cosmonauts wo are now routinely inhabiting the ISS. Naturally, the presence of humans also imposes the presence of their associated microorganisms in this confined habitat. Besides the arrival of new crew members roughly every six months and about one cargo transporter per month, delivering supplies and scientific equipment for experiments, the ISS is cut off from any other biological environment. Therefore, the ISS composes the most confined man-made and inhabited environment to date. In addition to its confinement, the ISS represents a very unusual microbial biotope. Higher radiation levels than on Earth, low

nutrient levels due to reduced introduction of new material, constant temperature (approx. 22°C), stable humidity (approx. 60%) and microgravity characterise the ISS habitat and make it a unique and extreme-situated indoor environment (Coil et al., 2016).

The microbiology on the ISS has been under surveillance since its first inhabitation. Standardised monitoring of surface and air samples onboard the ISS as well as more detailed post-flight investigations thereof have been conducted (Pierson, 2001; Castro et al., 2004; Alekhova et al., 2005; Novikova et al., 2006; Vesper et al., 2008; Satoh et al., 2011; Venkateswaran et al., 2014c; Checinska et al., 2015; Alekhova et al., 2016; Yamaguchi et al., 2016). Moreover, cleanliness of the ISS water supplies has been investigated (La Duc et al., 2003b; Bruce et al., 2005). The greater part of the first microbial investigations were mainly based on cultivation of bacteria and fungi on commercial high-nutrient media and under moderate conditions (Castro et al., 2004; Novikova et al., 2006; Van Houdt et al., 2012).

Since Roscosmos could observe serious problems due to microbial contaminations during operation of the space station Mir, all involved space agencies agreed on preventive measures to protect spacecraft, cargo, and crew from harmful microorganisms (e.g. (Novikova, 2004; Ott et al., 2014)).

For example, the air regeneration system is equipped with HEPA or equivalent filters (POTOK 150MK in Russian modules) to remove airborne microorganisms and particles ≥0.3µm. The acceptability limits for airborne bacteria and fungi were set to 10,000 and 100 CFUs/m^3 of air, respectively. For surfaces the respective limits were defined with 10,000 CFUs/100 cm^2 and 100 CFUs/100 cm^2. The microbial limits for the ISS water supplies differs between the US and the Russian segments: US water must be free of coliforms, with a total heterotrophic content of less than 100 CFUs/100 mL, while the Russian allow heterotrophic bacteria up to 10,000 CFUs/100 mL (Pierson, 2001; Van Houdt et al., 2012).

In order to avoid higher levels of microbial contamination, a rigorous housekeeping program is in place that includes weekly cleaning, biweekly disinfection and standard monitoring of ISS air and surfaces for viable bacterial and fungal contaminants every 90 days. The used disinfection agents are either based on a quaternary ammonium compound which is supplied by the US or on the combination of a quaternary ammonium compound with hydrogen peroxide, which is supplied by the Russians (Directorate, 2000; Pierson, 2001; Castro et al., 2004; Novikova et al., 2006; Duane et al., 2011; Van Houdt et al., 2012).

Monitoring of the microbial community onboard the ISS is highly important to evaluate material integrity of the spacecraft and to assess risk factors to the health of crew members. It

is known that the human immune system is compromised under space conditions. For example, there is a significant decrease of lymphocytes and also the activity of innate and adaptive immune response is reduced compared to terrestrial controls (Sonnenfeld and Shearer, 2002; Aponte et al., 2006). Additionally, it has been shown that the virulence of most microorganisms is affected by microgravity. For some species virulence is enhanced in space conditions (e.g. in *Salmonella typhimurium*) (Wilson et al., 2007) and reduced in the case of other species, such as *Listeria monocytogenes* or *Enterococcus faecalis* (Hammond et al., 2013). It is also debated that the efficacy of antibiotics and other medications decreases under space conditions (Taylor, 2015).

Even the integrity of the spacecraft itself can be compromised by microorganisms. So-called technophilic microorganisms, in particular fungi, are able to corrode alloys and polymers used in spacecraft assembly (Alekhova et al., 2005). These technophilic microorganisms caused major problems on the former Russian space station Mir (Novikova et al., 2001; Novikova, 2004). The main fungal genera detected onboard the ISS by cultivation were *Aspergillus* and *Penicillium* (Alekhova et al., 2005; Novikova et al., 2006; Venkateswaran et al., 2014c). These fungi were also found in higher abundance using different molecular approaches; however, Satoh et al. 2011 did not find any *Penicillium* in the Japanese Kibo module one year after its installation, but detected a predominance of skin-associated *Malassezia* (Satoh et al., 2011).

The main bacterial phyla detected onboard the ISS in air and on surfaces, by either cultivation or molecular methods, were Firmicutes and Actinobacteria. In cultivation-based assays, *Bacillus* and *Staphylococcus* species were the most detected Firmicutes, whereas signatures of *Staphylococcu*s utterly dominate the Firmicutes-affiliated signatures detected by molecular methods. The most probable reason for this observed discrepancy might be the disability of standard DNA isolation protocols to open spores adequately (Venkateswaran et al., 2014c).

This finding emphasises that cultivation approaches - although generally not able to record the whole diversity of a given environment (also stated above) - are still necessary for regular monitoring procedures. However, the ability of modern culture-independent molecular methods to assess the total microbial diversity present in a given environment is a powerful tool which enables researchers to elucidate the microbial community structure within the ISS beyond the standard cultivation assays. Next generation sequencing is nowadays also facilitating the microbiome analysis of the ISS. For instance, vacuum cleaner dust and filter debris collected from HEPA filters within the US American part of the ISS were analysed in

detail (Venkateswaran et al., 2014c) and their microbial inventory was also compared to the microbial inventory from spacecraft assembly cleanrooms (Checinska et al., 2015). Overall, there are several current projects which aim to broaden the knowledge about the ISS microbiome, including NASA´s "Microbial Observatory" project (Venkateswaran et al., 2014a), JAXA´s "Microbe" experiment series (Satoh et al., 2011; Ott et al., 2014; Yamaguchi et al., 2016) and ESA´s ARBEX project (Rettberg et al., 2016).

Almost all studies which investigated the ISS microbiome agree in one major aspect: the crew members act as the main source for the ISS microbial community, since most of the detected microorganisms are known to be human associated. The only studies which did not report a dominance of microorganisms of a presumable anthropogenic origin were studies conducted on the water supplies of the ISS, which is reasonable since these should normally not come in extensive physical contact with humans. Most of the organisms in the ISS water supplies were gram negative Proteobacteria, such as *Methylobacterium*, *Sphingomonas*, *Ralstonia* and *Pseudomonas* (La Duc et al., 2003b; Bruce et al., 2005).

Besides the human body, the other possible contamination source in this secluded habitat is the cargo delivered to the ISS including food, general equipment and material for scientific experiments. Cargo is always subjected to adapted cleaning procedures before upload and should be at least "visibly clean" before sent to the ISS (Pierson, 2001; Mord, 2009).

The crew on the ISS wears clothing, which does not impede the dispersal of microorganisms off the respiratory tract or skin and thus is certainly the major reason for the predominance of *Staphylococcus* (Firmicutes), *Corynebacterium* and *Propionibacterium* (Actinobacteria), which were also proven to be present in a viable status (Venkateswaran et al., 2014c).

Many human associated fungal and bacterial species are known to be opportunistic pathogens which are able to infect people with a (severely) compromised immune system. As mentioned above, the human immune system is proven to be compromised in space and the virulence of some (opportunistic) pathogens could even be enhanced under space-flight conditions. Additionally, if left uncontrolled in a confined environment where environmental strains are not present, which would normally outcompete human associated microorganisms under such conditions, human associated microorganisms can easily proliferate quickly and thereby pose a health hazard, as has been shown in artificial closed ecosystems on Earth (e.g. (Sun et al., 2016)). However, to date, there has been no serious infection reported on board the ISS, and the above mentioned CFU-limits were exceeded only in a few cases in which appropriate countermeasures succeeded in a timely manner (Van Houdt et al., 2012).

Taking all the publicly available information into consideration, and based on our current knowledge, one can conclude that the preventive measures which are in place on board the ISS are currently sufficient to ensure the safety of crew and spacecraft from the microbiological perspective. Nevertheless, the longitudinal analysis of microbial community behaviour under space conditions is necessary to deliver crucial knowledge to enable future long term space missions, as e.g. a flight to Mars and beyond.

Besides maintenance of a healthy environment and a healthy microbiome in the human body, the safe production of food and recycling of water has to be considered during long-term spaceflight. Spaceflight simulations, such as MARS 300 and MARS 500, and microbial monitoring thereof (Project: MICHA, DLR Cologne) are extremely helpful in order to elucidate potential pitfalls during a flight to Mars and beyond. However, much more research in this area is needed to ensure the health and well-being of the crew during such missions.

Recent and current studies on the overall microbial communities onboard the ISS help to understand the influence of inhabited microorganisms and reflect man-made environments on Earth (and *vice versa*). The overwhelming majority of detected microorganisms are, however, no threat towards human health or material but provide tremendous resources for human body function, sustainable waste remediation, recycling and purification of water and/or air supplies as well as nutrients for renewable food sources or even as a renewable food source themselves (e.g. (Nitta, 1999; Pierson, 2001; Czupalla et al., 2005; Bekatorou et al., 2006; Habib et al., 2008). In addition, the presence of beneficial microorganisms within a closed environment helps to suppress the harmful microbes and can thereby promote human health. As discussed in Mahnert et al. 2015, this could potentially be achieved by installing plants in such confined environments, which could support indoor air quality, mental health, provide a food source and support human's health and well-being by providing a natural microbiome source (Mahnert et al., 2015a).

Conclusion

Due to similar maintenance, architecture and type of confinement, the environments presented here habor a very specific microbial community. Intensive care units, operating rooms, cleanrooms and even the ISS share a number of typical microbial inhabitants, as displayed in Fig. 2. In particular *Bacillus* and human-associated microbial species are cultivated from all confined areas, reflecting the typical microbial community being composed of survival specialists (such as spore formers) and mainly representatives of the human microbiome, defining the human body as major source of microbial contamination.

The purposes for confinement are different. In the hospital area, the risk of infection is the major driving factor for confinement. Interestingly, higher efforts in cleaning (i.e. sterilization and bioburden reduction) do not decrease the risk for infections, in contrary: they were correlated with a higher incidence of infections and presence of multi-resistant strains. Parallels are found for cleanroom environments: the microbial inhabitants frequently showed higher resistances against physical and chemical stresses than their naturally occurring counterparts. All of these discussed areas are extreme and pose stresses towards the internal microbiome, which is then causing a reaction, namely adaptation and development of survival strategies.

Interestingly, the International Space Station seems to be a safe work space: despite allergic reactions (Venkateswaran et al., 2014c), so far no severe incidences of outbreaks have officially been reported. Certainly, although most confined, this is also the area with highest number and diversity of microorganisms acceptable, since neither persons, nor products are exposed to instantaneous risk.

Although cleanrooms are not living places for human beings, they have been subjected to comprehensive microbial analyses during the last years, using most sophisticated molecular and cultivation-based methods. While the overwhelming majority of cleanroom microorganisms appear to be dead, the survivors are specifically resistant and are considered possible contaminants of e.g. spacecraft targeting potential extraterrestrial biotopes.

In all areas, the routes of microbial transmission are not clearly resolved yet, leading to uncertainty with respect to optimal maintenance and risk management. Based on our experience and the information summarized in this review, we argue, that hygiene and maintenance strategies need to be critically reviewed, and the role of beneficial microorganisms, that naturally suppress unwanted microorganisms, need to be reassessed. The most-likely healthy transfer of beneficial microorganisms through e.g. pets or plants into patient rooms is currently restricted, due to uncontrollable risks. However, a controlled

spreading of selected, beneficial microorganisms in certain settings could help tremendously to improve quality of living and human health and to reduce long-term risks emanating from multi-resistant microbial strains.

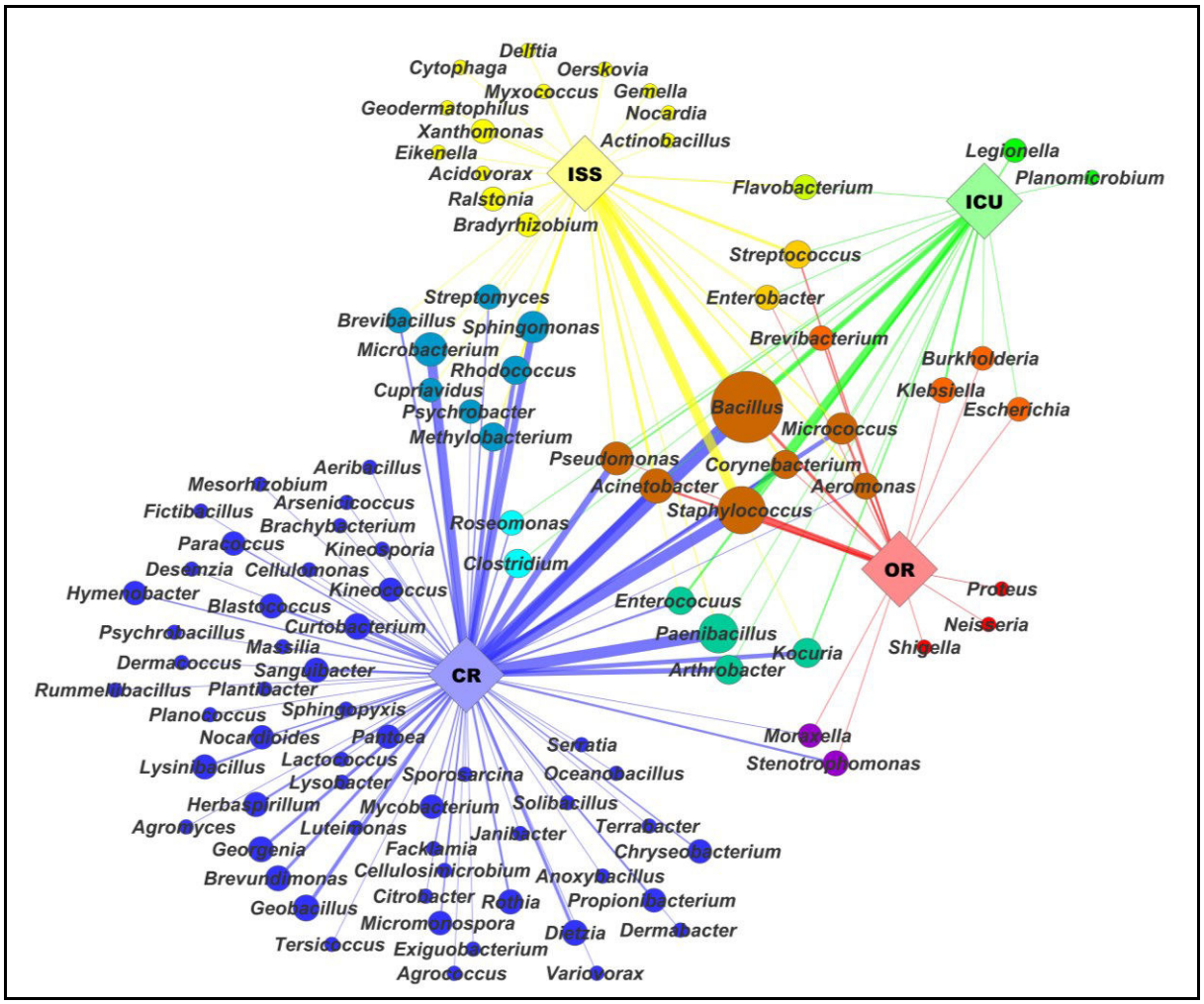

Fig. 2: The microbial network visualizes microbial profiles of selected confined habitats based on a range of isolates obtained from these environments. The network was arranged with Cytoscape using a spring-embedded algorithm on eweights. Diversity of isolates on genus level was correlated with node size. Nodes and edges were coloured by colour mixtures of their respective environments: international space station (ISS) – yellow, intensive care units (ICU) – green, cleanrooms (CR) – blue, and operating rooms (OR) – red. Edge width and opacity was correlated to respective eweights, which were computed in QIIME.

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Complex Indoor Communities: Bacterial Life Under Extreme Conditions in Clean Rooms and Intensive Care Units

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Synonyms

Microbiome of built environments

Definition

Indoor microbiomes are communities of microorganisms that inhabit the interior of built environments and are influenced by complex abiotic (e.g., climate, geographic location, building architecture, and maintenance) and biotic factors (human and animals/pets dynamics, greenery status, etc.).

Introduction to Indoor Microbiomes

Although microbes have often been recognized as pathogens, it is now well established that the majority of host-bacterial interactions are symbiotic (Blaser 2011). This partnership is based on molecular signaling to mediate beneficial outcomes for both microbes and their hosts.

Lisa Oberauner and Alexander Mahnert contributed equally

Complex Indoor Communities: Bacterial Life Under Extreme Conditions in Clean Rooms and Intensive Care Units, Fig. 1 Illustrations of built environments. (**a**) bedroom (private room), (**b**) office (public room), (**c**) intensive care unit (ICU), (**d**) spacecraft assembly clean room

This relationship between microbial diversity and host health was shown not only for plants and soils but also for animals and humans (Keesing et al. 2010). Despite the fact that the majority of our lifetime is spent in indoor environments such as the home, workplace, or public buildings (Fig. 1, Table 1), our knowledge of microbial diversity inside buildings is limited. We are not alone in these indoor environments: they provide new habitats and residence to numerous microbial communities comprising possibly hundreds of individual bacterial and fungal species. The most recent cultivation-based studies analyzed potential indoor pathogens with an emphasis on allergenic microorganisms (Yamamoto et al. 2011), yet little is known about the real microbial diversity indoors that has adapted to nutrient-poor, extreme conditions and communities that are composed of only a small fraction of cultivable microbes. The indoor microbiome should be continuously explored with special focus on the beneficial microbial inhabitants.

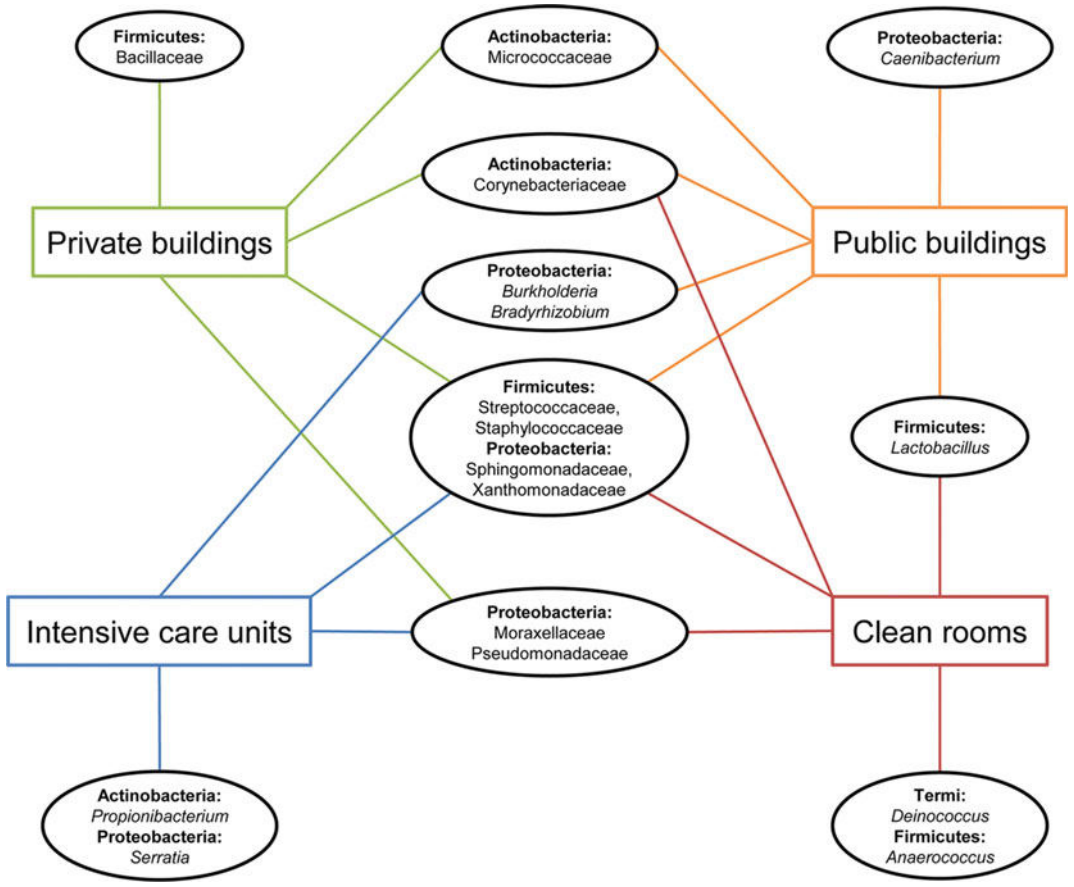
Complex Indoor Communities: Bacterial Life Under Extreme Conditions in Clean Rooms and Intensive Care Units, Table 1 Studies analyzing indoor environment microbiomes and parameters

Indoor environment	Classification	Human dynamic	Maintenance	Monitoring	Materials	References
Public buildings	Moderate	High (day), moderate (night)	Standard, mechanical ventilated	Moderate	Polymers, textiles, wood	Flores et al. 2011 Hewitt et al. 2012 Qian et al. 2012
Private buildings	Spare	Moderate (day), high (night)	Diverse, window ventilated	Low	Organic, wood, textiles, polymers	Flores et al. 2013 Dunn et al. 2013
Intensive care units (ICUs)	Strict	High (day and night)	Standard, mechanical ventilated, frequently cleaned, use of disinfectants, very sanitary	Controlled	Polymers, metals, textiles	Hewitt et al. 2013 Oberauner et al. 2013 http://hospitalmicrobiome.com/
Clean rooms	Strict	Minor (day and night)	Cleaning with alkaline reagents; controlling of particles, airflow, humidity, temperature; mechanical ventilated	Strict	Polymer, metals	La Duc et al. 2007 Moissl et al. 2007 Moissl-Eichinger 2011 Vaishampayan et al. 2013

Recently, the application of next-generation sequencing (NGS) techniques has provided new insights into indoor microbial communities (Fig. 2). In general, they are characterized by a high prokaryotic diversity and comprise diverse bacterial and archaeal phyla (Flores et al. 2011, 2013; Moissl-Eichinger 2011; Hewitt et al. 2012, 2013; Kembel et al. 2012; Kelley and Gilbert 2013). Indoor environments are also characterized by a specifically adapted fungal microbiome with an atypical building composition unlike those shown for bacteria (Pitkäranta et al. 2008). In addition, fungi are able to grow indoors when water is available (Zalar et al. 2011). Indoor microbiomes originate mainly from human skin, pets, or outside air and are even known to include extremophiles. Furthermore, all of them can contain potential human pathogens in addition to beneficial bacteria that are characterized by a positive interaction with their host (Flores et al. 2011; Kembel et al. 2012). Kembel et al. (2012) were the first to analyze patient rooms and find a strong impact from both architecture and ventilation. Similarly, other factors influencing the indoor diversity are of geographic and climatic origin (Hewitt et al. 2012). Two different types of microbial communities live indoors: airborne and surface-associated organisms. Airborne microbes – bacteria, fungi, or microscopic algae – are scattered and can travel long distances such as in the wind or in clouds before returning to the ground. Surface-associated microbes, however, tend to form biofilms. Despite the studies concerning indoor microbial communities published within the last 2 years in which molecular microbial ecology methods were applied, the majority of microbial coinhabitants in our built environments and their dynamics are still unknown.

The Impact of Indoor Microbiome on Human Health

Indoor microbial communities are an important component of everyday human health. They are partially composed of human-associated bacteria (Fierer et al. 2008) due to the high emission rate of up to 10^6 bacteria per person per hour, as reported from genome copies measured in the air from



Complex Indoor Communities: Bacterial Life Under Extreme Conditions in Clean Rooms and Intensive Care Units, Fig. 2 Overview of typical and dominant bacterial groups in the built environments. Schematic chart represents occurrence of the bacterial inhabitants indoors. Bacterial families and genera (*black ellipses*) are arranged according to their phylum affiliation (*bold*) and are connected to certain types of the built environments (*colored squares*). This image was compiled from the information in Table 1 and is not a holistic representation

individual persons (Qian et al. 2012). In hospitals and especially in intensive care units (ICUs), microbial spread can result in hospital-acquired "nosocomial infections" that compound underlying severe disease (Plowman 2000). Nosocomial infections remain among the leading causes of death in hospitals of developed countries, as the risk for nosocomial infections for patients in European ICUs, for example, was reported as 45 % (Plowman 2000). Hospital surfaces are often overlooked reservoirs for these bacteria (Kramer et al. 2006). Therefore, new sanitation standards are needed to drastically reduce the risk for these hospital-acquired infections. Indoor microorganisms also affect human health as allergenic agents (Hanski et al. 2012). They are also involved in the development of the sick building syndrome (SBS), which causes symptoms such as sensory irritation of the eyes, nose, and throat; neurotoxic or general health problems; skin irritation; nonspecific hypersensitivity reactions; and odor and taste sensations.

Bacterial Communities in Intensive Care Units

In contrast to the majority of indoor environments, rooms in hospitals and especially in

intensive care units (ICUs) are routinely monitored for the presence of microbes (Fig. 1, Table 1) (Hewitt et al. 2013). However, this monitoring is based on microbial cultivation and not DNA sequencing. In a recent study, 16S rRNA gene amplicon pyrosequencing was used to study the ICU microbiome in comparison with the currently used standard cultivation technique (Oberauner et al. 2013). Only 2.5 % of the total bacterial diversity was detected using cultivation; however, all sequences were represented in the sequencing libraries. The phylogenetic spectrum comprised 7 phyla and 76 genera and included species associated with the outside environment, taxa closely related to potential human pathogens, and others belonging to beneficial organisms. Specifically, *Propionibacterium*, *Pseudomonas*, and *Burkholderia* were identified as important sources of infection (Fig. 2). Despite significantly different bacterial area profiles for floors, medical devices, and workplaces, network analysis and molecular fingerprints were used to show similarities and evidence for the transmission of strains. This information allows for a new assessment of public health risks in ICUs and will help to create new sanitation protocols to better understand the development of hospital-acquired infections.

Bacterial Communities in Clean Rooms

Clean rooms are established facilities that have been involved in various industrial processes since the 1940s (Fig. 1). Whereas clean rooms were first applied in the areas of microtechnology and optics, today they are used for the production of semiconductors and in medical, pharmaceutical, and food production, as well as in spacecraft assembly. Clean rooms are classified by the numbers and sizes of particles allowed within them. For the DIN EN-ISO 14644-1 classification, the ISO classes 1–6 correspond to the number of particles ($10–10^6$) per m^3 with 0.1–0.2 μm in size. The amount of these particles is controlled via filters, airflow rate, pressure, humidity, and temperature. Despite stringent cleaning and maintenance, clean rooms used for spacecraft assembly are not devoid of microorganisms, and many hardy extremophiles can survive in these oligotrophic conditions (Table 1) (La Duc et al. 2007; Moissl et al. 2007; Moissl-Eichinger 2011). Due to planetary protection regulations, a peculiar monitoring of biological contaminants (bioburden) and characterization of the microbial populations in the well maintained, extremely low-biomass environment must be followed at each step of the assembly process. Most of the standard assays are based on cultivation-dependent methods; however, there has been a recent trend to also include cultivation-independent methods that include genomic approaches (Vaishampayan et al. 2013).

Bacterial communities in the spacecraft assembly clean rooms at the EADS facility in Friedrichshafen (Germany) and at the NASA Jet Propulsion Laboratory (JPL, CA, USA) were investigated in a joint project. Floor samples were studied using cultivation-dependent (mesophiles/oligotrophs, alkaliphiles/alkalitolerants, and facultative anaerobes) and cultivation-independent assays [ATP assays and propidium monoazide (PMA) pretreatment PCRs] to measure microbial burden (Vaishampayan et al. 2013). When samples were pretreated with PMA prior to DNA extraction, the chemical intercalated into DNA from dead microbes, thus disabling PCR amplification (Wagner et al. 2008). The PMA-pretreated (viable microbes) and untreated (total microbes) portions were analyzed using quantitative PCR (qPCR) and 16S rRNA gene amplicon deep sequencing to estimate bioburden and to measure viable microbial diversity, respectively. Overall, the clean room floors contained less total and viable microbial burden when measured by any assay than the adjacent servicing area locations. Hence, stringent maintenance and cleaning reduced the viable microbial population in the clean room by 1–2 orders of magnitude. This reiterates the fact that the proper maintenance of the NASA JPL spacecraft assembly clean room floors removed substantial numbers of microbial cells, but some selective microbial populations were able to survive under these clean conditions. ATP assays and PMA-qPCRs are both suitable to target the viable microbial population. However,

the deep sequencing analysis in combination with a prior PMA treatment showed that viable microbial diversity also exists in the clean room and not only in the servicing area as expected. While Proteobacteria and Firmicutes were the dominant bacterial phyla (Fig. 2), Archaea and fungi were also detected. Most microbes seem to be introduced by humans. In addition, a metagenomic approach targeting various genes is planned at JPL to reveal the presence of active functional microbial species. Results of this study will enable scientists to accurately track the true viable microbial population and perform accurate risk assessment of microbial contamination to the assembled products in the clean room environment.

Summary and Conclusions

Indoor microbiomes are complex microbial ecosystems influenced by diverse abiotic and biotic factors. Indoor microbes originate from humans, pets, indoor and outdoor plants, dust, and soil; altogether every individual leaves a significant signature within his or her built environment as a result of unique microbiomes and activities. Advances driven by novel high-throughput technologies (e.g., next-generation sequencing) have completely altered our perspective on the microbiology of built environments. Therefore, these techniques should also be used not only for the evaluation of standard maintenance in clean rooms and validation of clean room products but also for the evaluation of our hygiene standards in hospitals. Overall, the indoor microbiome plays an important role for human health and includes both pathogens and a substantial proportion of beneficials, which should be ultimately maintained.

Cross-References

- ▶ Fungus in the Human Microbiome, Definition and Examples
- ▶ Human Microbiome Project, Reference Genomes, Rationale, Selection, Acquisition, Sequencing, and Annotation
- ▶ Methanogenic Archaea in the Human Microbiome
- ▶ New Tools for Understanding, Composition and Dynamics of Microbial Communities, Project

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Computational Tools for Taxonomic Assignment

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Introduction

Classification of bacteria and the definition of bacterial taxonomies themselves were both historically based on phenotypic attributes of cultured isolates or whole genome DNA-DNA hybridization. More recently, molecular sequencing methods form the basis of most classification approaches used in metagenomics, and modern bacterial taxonomies are more explicitly defined according to phylogeny (Hugenholtz 2002). Computational tools for classification of bacterial DNA sequences can be roughly categorized on the basis of the means of sequence comparison: classification may be performed on the basis of primary DNA sequence homology, phylogenetic criteria, composition (i.e., attributes other than primary DNA sequence such as oligonucleotide frequency), or some combination thereof (Bazinet and Cummings 2012). Another axis upon which to categorize classification tools is whether they use specific gene targets ("marker genes") or unspecified genomic fragments. The most commonly used marker gene, and the current gold standard for classification and phylotyping in metagenomic surveys, is the 16S rRNA gene, but others, either in isolation or in combination, are in use as both universal targets or in a more limited taxonomic context. For example, *rpoB* can been used to resolve a range of bacterial groups (Case et al. 2007), and the *hsp65* gene is commonly used for classification of mycobacterial species (McNabb et al. 2004). It is important to consider that regardless of the classification method, results are heavily influenced by the completeness and accuracy of the database of reference genomes or marker gene sequences. For this reason, evaluation of the relative performance of each tool is challenging from the literature.

Classification on the Basis of Primary DNA Sequence Identity

One strategy to obtain high-resolution classification is on the basis of primary DNA sequence homology: algorithms using this strategy consider pairwise alignments between query and reference sequences, and transfer the taxonomic name of a reference sequence onto the query if the pairwise identity, E-value, or some other measure of similarity, exceeds some threshold value at a given rank. For example, criteria for classification using 16S rRNA gene sequence using pairwise identity at the species level range from 98 % to 99.5 % (Clarridge 2004). Lower identity thresholds may be used at less specific ranks (e.g., Hummelen et al. 2010). Reference databases may be comprehensive, for example,

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Quantification of Encapsulated Bioburden in Spacecraft Polymer Materials by Cultivation-Dependent and Molecular Methods

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Abstract

Bioburden encapsulated in spacecraft polymers (such as adhesives and coatings) poses a potential risk to jeopardize scientific exploration of other celestial bodies. This is particularly critical for spacecraft components intended for hard landing. So far, it remained unclear if polymers are indeed a source of microbial contamination. In addition, data with respect to survival of microbes during the embedding/polymerization process are sparse. In this study we developed testing strategies to quantitatively examine encapsulated bioburden in five different polymers used frequently and in large quantities on spaceflight hardware. As quantitative extraction of the bioburden from polymerized (solid) materials did not prove feasible, contaminants were extracted from uncured precursors. Cultivation-based analyses revealed $<$0.1–2.5 colony forming units (cfu) per cm^3 polymer, whereas quantitative PCR-based detection of contaminants indicated considerably higher values, despite low DNA extraction efficiency. Results obtained from this approach reflect the most conservative proxy for encapsulated bioburden, as they give the maximum bioburden of the polymers irrespective of any additional physical and chemical stress occurring during polymerization. To address the latter issue, we deployed an embedding model to elucidate and monitor the physiological status of embedded *Bacillus safensis* spores in a cured polymer. Staining approaches using AlexaFluor succinimidyl ester 488 (AF488), propidium monoazide (PMA), CTC (5-cyano-2,3-diotolyl tetrazolium chloride) demonstrated that embedded spores retained integrity, germination and cultivation ability even after polymerization of the adhesive Scotch-Weld 2216 B/A. Using the methods presented here, we were able to estimate the worst case contribution of encapsulated bioburden in different polymers to the bioburden of spacecraft. We demonstrated that spores were not affected by polymerization processes. Besides Planetary Protection considerations, our results could prove useful for the manufacturing of food packaging, pharmacy industry and implant technology.

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Introduction

In course of space exploration, the potential danger to contaminate celestial bodies with terrestrial microorganisms (or *vice versa*) has received growing attention. The goal of planetary protection is to mitigate such risks, which are most severe in case of missions to planets where conditions are favorable for life. In order to reduce the risk of contamination and ensure the scientific value of possible life detection missions, the Committee on Space Research (COSPAR) has issued specific regulations for spacecraft cleanliness [1]. Spacecraft under planetary protection limitations are constructed in clean rooms and maintained under bioburden control to diminish risks of microbial contamination. Sampling of the spacecraft-associated microbiome (including microbial heat shock resistant bioburden) is employed to study the microbial diversity of the cleanroom environment, spacecraft elements, and the assembled spacecraft (e.g. [2,3,4,5,6,7,8]). Airborne contamination is collected by active sampling (the most commonly used samplers in the pharmaceutical and medical device industry are impaction and centrifugal samplers [9]), while bioburden on surfaces can be detected by the use of swabs, wipes, or similar devices [10,11,12]. However, these sampling procedures cannot cover all sources for microbiological contamination. A potential contamination pathway that escapes such standard controls is the bioburden encapsulated in polymers, which are widely applied in the construction of spacecraft [13].

For modern spacecraft hardware lighter but often more porous composite materials are preferred over those heavy stainless steel materials used in the Viking era. Beside metal-based materials, spacecraft hardware comprises silicones (e.g. in electronic components, optics, and solar cells), other polymeric materials (e.g. parachutes, airbags, thermal covers, coatings, paints, and wires) and carbon-polyesters used for structures. Due to their porous structure these new materials increase the risk of an accidental contamination of extraterrestrial environments [14].

Bacterial spores embedded or encapsulated in these pores are – to a certain degree – protected from stresses like space vacuum and radiation during space travel and from heating during entry into a planet's atmosphere [15,16,17,18,19,20,21,22,23]. Such sheltered bioburden has to be considered, since materials degrade, and some parts of a spacecraft intended to land on a planet's surface may break (e.g. the head shield, the back shell, parts of the active descent system such as engines). In case such impacts do not result in complete sterilization of spacecraft hardware due to entry heating, they may have some potential to inoculate microbes into favorable environments of a planet's bottomset beds [24,25].

The risk of bioburden enclosure in polymers is not equal for all materials. Structural materials like polyimides are cured at high temperature conditions of several hundred °C, which are not survived even by the most resistant spore-forming microorganisms known. Other polymers are used in spacecraft parts which are treated under very stringent conditions, e.g. the heat shield, which is degassed for extended periods at elevated temperatures. Some polymers are cured from organic precursors, which are microbicidal to vegetative microorganisms but not spores. Some, polymers contain fillers or strengthening enclosures that may carry a different microflora than the encapsulating polymer itself and may protect embedded bioburden.

For the purpose of this study, "encapsulated bioburden" was defined as heat-shock resistant bioburden inside (i.e. not free for gas/water vapor exchange) non-metallic materials. This includes microorganisms physically surrounded by and in direct contact with the polymer matrix, and those indirectly surrounded by polymer in pores, bubbles or protected by non-polymeric materials (e.g. fillers, insulating or reinforcing structural components).

Detection of encapsulated microorganisms in cured polymers is a challenging task, because harsh and destructive extraction methods are needed to release microorganisms from these materials. In a recent study [26] *Bacillus pumilus* SAFR-032 spores were artificially encapsulated in poly(methylmethacrylate) (Lucite, Plexiglas) and released by dissolution in organic solvents. However, for most other polymers no effective solvents are available. In the present communication, we used Scotch-Weld 2216 B/A adhesive as a model polymer to determine the survivability of purposely encapsulated microorganisms, to investigate the effectiveness of spore recovery from uncured precursor, and to estimate the intrinsic bioburden in polymers. The aim of this study was to develop cultivation- and molecular-based methods for assessing the microbial bioburden within polymeric materials and thus be able to estimate their quantitative contribution to the total bioburden of spacecraft.

Materials and Methods

Bacterial Strains and Cultivation Conditions

Bacterial strains were purchased from the Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). *Bacillus safensis* DSM 19292^T was selected as the standard spore model for most experiments due to its high resistance to various environmental stresses and as an obvious contamination source of spacecraft and clean room environments (*B. safensis* was isolated from the surface of the Mars Odyssey Orbiter spacecraft and also the assembly-facility surfaces at Jet Propulsion Laboratory and Kennedy Space Center [27]). Available spore preparations revealed a high number of germinable spores, (17.6%) in contrast to *Geobacillus stearothermophilus* (< 5%) used only for visualization of spores in the embedding model (see below). Hence, the number of viable but non-germinable spores (VBNG) was kept low. Spore suspensions were prepared by lysozyme (200 ng/µL) and DNase treatment followed by heat shock (15 min, 80°C) as described previously [28,29]. For the experiments, *B. safensis* was incubated for 2–5 d at 32°C on soybean casein digest agar (Becton Dickinson, Heidelberg, Germany). Spores of *Geobacillus stearothermophilus* DSM 5934^T were collected from agar medium and incubated in 65% 2-propanol for 3 h (at room temperature), followed by two wash cycles with deionized water. *G. stearothermophilus* was incubated 2–5 d at 55–60°C on soybean casein digest agar.

Polymer Materials

The analyzed materials for flight hardware construction and their preparation are listed in **Table 1**. For each material, the most suitable solvent for dissolution or dilution of the uncured polymer was determined by mixing precursors with different solvents (at concentrations of 10–50 wt.-%) and vigorous vortexing. The samples were incubated up to 24 h at room temperature to observe if sedimentation or polymerization occurred.

Surface-embedding of Spores in Scotch-weld 2216 B/A

B. safensis or *G. stearothermophilus* spores were washed twice in acetone and diluted to obtain an acetonic spore suspension of $\sim 1 \times 10^5$ colony forming units (cfu)/mL. One drop of the suspension was spread on the surface of water-soluble polymer (poly vinyl alcohol (PVA)) and dried for 2 d at room temperature. 1–2 g of freshly prepared Scotch-Weld 2216 B/A (**Table 1**) were placed on top of the dried spore suspension on PVA. The polymer was allowed to cure for 7 d at room temperature. The water soluble PVA was peeled off or washed from the sample after curing, leaving embedded spores concentrated in a layer proximal to the surface of the material.

Scanning Electron Microscopy (SEM) and Dual Beam Focused Ion Beam (FIB)

Samples of inoculated surfaces of the adhesive Scotch-Weld 2216 B/A were prepared on a dual-beam FIB/SEM workstation (FEI Helios Nanolab) and investigated by field emission scanning electron microscopy (FESEM) using a Hitachi SU-4800 instrument operated at 1 kV. Prior to inserting the sample in the FIB, a thin Au layer was sputter-deposited on the surface of the sample to protect the polymer in the subsequent preparation steps. FESEM scanned the specimen in a grid to create an image. FIB cut out a 10 µm by 10 µm test area with an ion beam perpendicular to the specimen surface, which was then sectioned by the ion beam in 1 µm steps. Images were taken by an SEM vertically oriented to the ion beam. FESEM images of PVA, and inoculated and non-inoculated Scotch-Weld 2216 B/A allowed visualization of embedded spores in the adhesive. FIB/SEM images verified the partial embedding of the spores necessary for further staining approaches (see **Fig. 1**).

Table 1. List of materials analyzed, their preparation, and the solvent most suitable for dilution of the uncured precursors.

Name (Supplier)	Type	Utilization in spacecraft	Preparation	Solvent used for extraction	Density (g/cm^3)
Scotch-Weld 2216 B/A (3M, France)	Epoxy adhesive	screw locks, bonding	component A (hardener): component B (base) = 7:5 (w/w)[1]	2-propanol	1.32
SG121FD (MAP, France)	Silicone coating	Thermal control paint	base : catalyst = 86:14 (w/w)	MAP SG121FD thinner	1.41
Solithane 113 (Specialty Polymers & Services, USA)	Urethane resin	conformal coating, screw locks, bonding, insulation	S113 : C113–130 (hardener) = 100:70 (w/w)[2]	ethanol	1.07
ESP 495 (ACC Silicones/EADS Astrium)	Silicone adhesive	Adhesive for thermal protection system	–	acetone	1.07
Dow Corning 93–500 (Ellsworth Adhesives GmbH, Germany)	Silicone encapsulant	Sealing and bonding	encapsulant : curing agent = 10:1 (w/w)[2]	acetone	1.08

[1]mixed thoroughly for 5 min.
[2]mixed thoroughly for 1–2 min.
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Fluorescence-based Detection of Surface-embedded Spores and Confirmation of their Identity after Re-growth

Detection of (partially) embedded, intact spores in Scotch-Weld 2216 B/A was performed by staining spores with AlexaFluor succinimidyl ester 488 (AF488, MoBiTec, Göttingen, Germany) prior to the embedding procedure and with propidium monoazide (PMA, Biotium, Hayward, CA, USA) subsequent to embedding. AF488 is a fluorescent dye that can be applied for the visualization of many vegetative cells [30] as well as spores [29]. For labeling, spores were exposed to 100 µl ATTO-dye labeling buffer (ATTO-TEC GmbH, Siegen, Germany), to yield unprotonated amino groups of proteins, and 10 µg AF488 were added. Reaction between dye and proteins was enabled at RT in the dark on a shaker for 30 min. Stained spores were washed three times with sterile water to remove unspecific staining. PMA selectively enters wall-compromised cells and binds to DNA [31] as has also been demonstrated for heat-inactivated *B. safensis* spores [29]. After treatment of spores with 10 mM dithiothreitol (DTT) at 65°C for 15 min (to increase permeability), they were stained with 50 µM PMA in the dark for 50 min. Cross-linking of PMA with DNA was enabled via halogen light (500 W) exposure for 3 min of samples kept on ice. The labeling procedure was completed by three serial washing steps. Cy5 (cyanine dye 5) was applied as a surface marker, by adding 2 µL of an aqueous dye stock solution (30 µM) on top of the spore preparation.

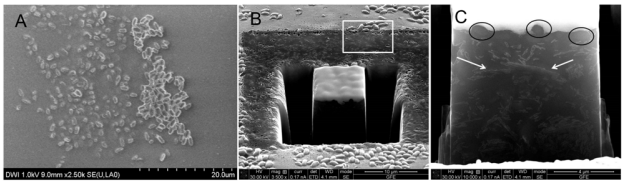

Figure 1. Surface embedded spores of *G. stearothermophilus* in cured Scotch-Weld 2216 B/A. (A) Scanning electron micrograph. Spores on the right side of the image appear sharply outlined and are apparently either surface-attached or partially embedded and protruding from the surface of Scotch-Weld 2216 B/A. Spores on the left side of the image appear more deeply embedded below the surface. (B) Overview of embedded spores at starting position of dual beam FIB/SEM measurement. The rectangle shows the area where the FIB ablation was performed to image the sample cross section. Spores partially embedded in the surface of cured Scotch-Weld 2216 B/A are visible. During sectioning of the samples with FIB, which removes layer after layer of 1 µm thickness each, vertical sections can be visualized by SEM to obtain a side view of the embedded spores in the adhesive (Fig. 1, C). (C) The outline of spores embedded in or near the surface of Scotch-Weld 2216 B/A can be seen along the cross section. Spores are partially or fully embedded (circles). Kaolin (filler material in Scotch-Weld 2216 B/A) inclusions are also visible (marked by arrows).
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50 mM CTC (5-cyano-2,3-diotolyl tetrazolium chloride, Polysciences Europe GmbH, Eppelheim, Germany) was used to visualize metabolic activity of *B. safensis* during its germination process. Spore germination was provoked by incubation in growth medium or glucose solution (at 32°C, approx. 1–2 h; either in tubes or for embedding models covered with growth media and incubated horizontally). Slightly attached and not properly embedded spores were removed by sonication at maximum intensity (120 W, 35 kHz) for 10 min in a 50 mL falcon tube filled with 35 mL of sterile distilled water to guarantee evaluation of embedded or encapsulated spores. Fluorescently labeled spores were monitored via confocal laser scanning microscopy (CLSM) as described previously [29], using an inverse Laser Scanning Microscope (LSM 510-Meta confocal microscope, CLSM, Zeiss, Munich, Germany). Fluorescence signals of PMA or CTC (excitation 514 nm, detection 505 nm), AF488 (excitation 488 nm, detection 505–530 nm) and Cy5 (excitation 633 nm, detection 650 nm) were detected in the multi-track mode to avoid cross-talk phenomena. Z-stacks were scanned with a 50% overlap of each 0.4 µm section. CLSM images were analyzed, arranged and 3D projections were made with the Zeiss LSM Image Browser Software.

CLSM monitored embedding models were subjected to cultivation dependent analysis to support conclusions drawn from fluorescence staining of embedded spores. Scotch-Weld 2216 B/A surfaces were cleaned with 70% (v/v) ethanol to minimize contaminations caused by non-sterile CLSM procedures. For cultivation 1 mL tryptic soy agar was poured on model surfaces and incubated at 32°C for 4 d. Grown colonies were identified (Geneart Life Technologies, Regensburg, Germany) by their PCR amplified *rrnB-16S* and *gyrB* gene sequences (as *B. safensis* and *B. pumilus* show 99.9% identity on *rrnB-16S* gene level but show adequate differences (91.2% identity) in their respective *gyrB* genes [27]. Bacteria-specific primers 9 bF (GRGATCCTGGCTCAG) [32] and 1406uR (ACGGGCGGTGTGTRCAA) [33] (1.25 ng/µL each) were used for *rrnB-16S* gene PCR containing 200 µM dNTP mix, 2 U of *Taq* DNA polymerase in Buffer Y with $MgCl_2$ (10x). DNA templates were amplified in 10 cycles of denaturation

at 96°C for 30 sec, annealing at 60°C 30 sec, elongation at 72°C for 1 min, followed by 25 cycles of a changed denaturation at 94°C for 20 sec. For *gyrB* gene targeted PCR 1 µg of template DNA was amplified with UP-1long (GAAGTCATCATGACCGTTCTGCA(TC)GC(TCAG)GG(TCAG)GG(TCAG)AA(AG)TT(TC)AG) and UP-2r (AGCAGGGTACGGATGTGCGAGCC(AG)TC(TCAG)AC(AG)TC(TCAG)GC(AG)TC(TCAG)GTCAT) primers [34,35] (1 µM each). PCR settings were adjusted to the following conditions: 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C 1.5 min, and elongation at 72°C for 2.5 min. PCR sequences were compared with GenBank deposited sequences (http://www.ncbi.nlm.nih.gov/genbank/) using the basic local alignment search tool (blastn; http://blast.ncbi.nlm.nih.gov/Blast.cgi) [36].

Survival of *B. Safensis* Spores in Uncured Polymer Precursors

To determine the possible sporicidal effects of the uncured polymer precursors, a *B. safensis* spore suspension (10^7 cfu/mL) was washed twice in its respective solvent (depending on the material tested, see **Table 1**). 0.5 g of freshly-prepared polymer was mixed with the final spore pellet and incubated 0–60 min at room temperature. After adding 1 mL of solvent to the polymer-spore mixture and vigorous mixing, the resulting suspension was serially diluted in sodium chloride peptone buffer. The appropriate dilutions were subjected to heat shock (80°C, 10 min, according to specifications of US Pharmacopeia [37]) and pour-plated on soybean casein digest agar. For comparison the cultivable spore number after washing and resuspending in solvent without polymer was determined. For these experiments, growth conditions were always identical for experimental and control samples. It was not attempted to optimize recovery conditions for *B. safensis*, as this organism served as model to comparatively evaluate the effect on spore survival of the polymer precursors. Recovery of viable spores after incubation was always lower than the inoculum, which is to be expected due to losses during the experimental procedures, but generally high enough to reasonably expect spores to survive in polymer precursors.

Recovery of *B. Safensis* Spores Grown in the Presence of Polymer Precursors on Nutrient Agar

Components of the polymer materials were prepared as shown in **Table 1**, diluted in the appropriate solvent (0.1–0.5 g/mL, depending on the solubility of the precursors), and inoculated with *B. safensis* spores to reach a final spore concentration of 10^2 cfu/mL. *B. safensis* spores (10^2 cfu/mL) in solvent only (without the polymer precursors) served as a positive control. Samples were subjected to heat shock (80°C, 10 min) to activate endospores. 1 mL aliquots were pour-plated in soybean casein digest agar. Plates were incubated 5 d at 30–35°C to detect inoculated spore-formers and to check if residues of the polymer precursors inhibited germination and growth. All colonies appeared within 2 days, no additional growth was observed when incubation was prolonged. All our solvent extraction methods achieved recovery rates above 10% of the positive controls and were thus considered suitable.

Cultivation-based Recovery of Intrinsic Endospore Burden from Polymer Precursors

Components of the polymer materials were prepared as shown in **Table 1**, and diluted in the appropriate solvent (0.1–0.5 g/mL, depending on the solubility of the precursors). The method was optimized for detection of cultivable bacterial endospores by including a heat activation step before pour-plating 1 mL aliquots on soybean casein digest agar or R2A agar (Sigma-Aldrich; Steinheim, Germany). Plates were incubated 5–7 d at 30–35°C, followed by 5–7 d at 55–60°C to detect mesophilic and thermophilic spore-formers, respectively. In total, 45–80 cm^3 of each material was investigated. If colonies were detected, they were isolated and visualized microscopically after Gram-staining.

Calculation of the Encapsulated Bioburden in Precursors Corrected for the Recovery Efficiency

To calculate the corrected value for mean encapsulated bioburden (CME), the recovery efficiency was included in the determined colony number.

Recovery efficiency (RE) was calculated by

$$RE = \frac{MR}{MI}$$

with a standard deviation of $sd(RE) = RE\sqrt{\left[\left(\frac{sd(MR)}{MR}\right)^2 + \left(\frac{sd(MI)}{MI}\right)^2\right]}$.

Here: MR = mean recovered spores from the polymer precursors, MI = mean inoculated spores, and calculated standard deviations sd(MI) and sd(MR).

Tests for bioburden were repeated 4–10 times for each material, yielding mean and standard deviation of the number of colonies recovered from a certain volume of material (ME, sd(ME).

Mean encapsulated bioburden $CME = \frac{ME}{RE}$, with standard deviation:

$$sd(CME) = \frac{ME}{RE}\sqrt{\left[\left(\frac{sd(ME)}{ME}\right)^2 + \left(\frac{sd(RE)}{RE}\right)^2\right]}$$

The values given in this report are conservative values, calculated by $CME + 1sd(CME)$. When 0 cfu were recovered, calculations were performed with 1 cfu, and CME was given as less than (<) the resulting value.

Cultivation-based Determination of Bioburden in Kaolin

To determine the bioburden of kaolin (filler material of Scotch-Weld 2216 B/A), samples of kaolin in 3 grain sizes (ASP 200, 600, and 900) were obtained from a supplier in Germany (Quarzwerke, Frechen, Germany). Of each grain size, 10 g were suspended in duplicate in 90 mL sodium chloride peptone buffer (Merck kGaA, Darmstadt, Germany, prepared according to manufacturer's instructions) and subjected to heat shock (80°C, 10 min) or incubation at room temperature in parallel. Of each sample, 0.1 mL in 10 replicates was spread-plated on R2A agar, and plates were incubated for 7 d at 30–35°C. The respective contribution of kaolin to the weight of the adhesive was taken into account (8.9% for ASP 200, and 11.4% for ASP 600 and ASP 900 each) to determine the total bioburden of Scotch-Weld 2216 B/A.

DNA Extraction from Kaolin and Purification

DNA was extracted from kaolin (filler in Scotch-Weld 2216 B/A) with the Precellys Bacterial/Fungal DNA kit (Peqlab, Erlangen, Germany). 0.1 g kaolin (of three grain sizes: ASP 200, 600 and ASP 900; in triplicates) was suspended in 200 µL Tris-EDTA (TE) buffer. Suspensions were transferred to a 2 mL Precellys tube and processed as indicated by the manufacturer. The extracted DNA (solved in 50 µL Elution Buffer) was purified by precipitation overnight at −20°C with the same volume of ice-cold 2-propranol (abs.), followed by washing with ice-cold ethanol (70% v/v) and

drying of the pellet for 3 h. DNA was resuspended in 15 µL PCR-grade water and stored at −20°C till further use. DNA-free extraction blanks for qPCR experiments were prepared by suspension of a mixture of ASP 200, ASP 600 and ASP 900 (0.033 g each, triplicates) in 1 mL of sterile DNaseI buffer (100 mM Tris-HCl; pH 7–7.5; 25 mM $MgCl_2$; 5 mM $CaCl_2$). 2 µL of DNaseI (1 mg/mL) were added to the kaolin suspension to digest present DNA by incubation at 37°C for 1 h. The enzyme was inactivated at 90°C for 1 h, and removed by washing three times with sterile water. After 2 d of drying at room temperature, the extraction blanks were subjected to the DNA extraction and purification procedure as described above.

DNA Extraction from Polymer Precursors

Samples were prepared by dissolving 5 g Solithane 113 in 10 mL ethanol (abs.). Ethanol inoculated with *B. safensis* spores (2×10^6) was used as a positive control, and the same volume of ethanol was processed as extraction negative control. Samples were concentrated by centrifugation (8,600×g, 1 min) and resuspension of the pellet in 100 µL TE buffer; however, the formed precipitates remained undissolvable. For decoating of spores, 500 µL decoating buffer (50 mM Tris Base (pH>9.5), 1% (w/v) sodium dodecyl sulfate (SDS), 8 M urea, 50 mM dithiothreitol (DTT), 10 mM Na_2EDTA) were added, followed by incubation at 60°C for 90 min with shaking [38]. Samples were washed three times with Sodium chloride-Tris-EDTA (STE) buffer (10 mM Tris-HCl (pH 8), 10 mM Na_2EDTA, 150 mM NaCl) and once with Lysis buffer (Precellys Bacterial/Fungal DNA kit, Peqlab). Afterwards, pellets were resuspended in 300 µL Lysis buffer and stored overnight at −20°C. After homogenization of the samples and degradation of cells, following the manufacturer's instructions as described above (see DNA extraction from kaolin), the suspensions were transferred to peqGold PhaseTrap columns (Peqlab) after short centrifugation (12,000×g, 20–30 sec). The same volume of 24:25:1 (v:v:v) chloroform:phenol:isoamyl-alcohol was added and carefully mixed, followed by centrifugation at 1,400×g rpm, 5 min, at room temperature. Subsequently, the supernatant was transferred into a new tube and mixed with 0.057 vol. 7 M ammonium-acetate and 1 vol. of ice-cold 2-propanol (p.a.). For precipitation, the mixture was stored at $-20°C \geq 4$ h before washing with 1 mL 70% (v/v) ethanol (16,900 rpm, 30 min, 4°C). The supernatant was discarded and the pellet was dissolved in 15 µL water after 1 h drying at room temperature.

Quantitative PCR (qPCR)

Reaction mix for each sample (final volume 20 µL) consisted of 10 µL SYBR Green mix (Qiagen, Hilden, Germany), 1 µL forward (338 bF, ACTCCTACGGGAGGCAGCAG) and reverse primer (517 uR, GWATTACCGCGGCKGCTG; 25 ng/µL each)) [33,39], 7 µL H_2O and 1 µL template (DNA sample or extraction blank or water (negative control)). Prepared qPCR suspensions were gently mixed and tubes were loaded into the Rotor Gene 6000 Cycler. The PCR program was adapted from the SYBR Green protocol (Qiagen) with a hot start step for polymerase activation at 95°C for 15 min and 40 cycles of 94°C for 15 sec, 60°C 30 sec, 72°C 30 sec. qPCR amplification was terminated with a melt curve from 72°C to 95°C. For analysis of the amplification results, the Rotor Genes 6000 software was used. 1 µL of the *rrnB-16S* gene PCR product of *Bacillus atrophaeus* was included as a standard (10^4 to 10^6 *rrnB-16S* gene copies). Since DNA templates extracted from kaolin interfered with the qPCR, one standard dilution was mixed with a DNA-free extraction blank from kaolin in order estimate the effect on the amplification process. The inhibitory effect in kaolin samples was calculated according to differences between the kaolin extraction blank + standard and the pure standard. This value was used to correct kaolin sample values without an inhibiting effect. Values were equalized to 1 g kaolin and the estimated copy numbers were divided by a mean of all known bacterial *rrnB-16S* gene copy numbers per cell (4.1 *rrnB-16S* gene copies per cell; source: ribosomal database rrnDB [40,41]).

Results

General Remarks and Test Strategy

The test strategy to determine encapsulated bioburden was developed with Scotch-Weld 2216 B/A as an example polymer, because it combined several relevant features. The physical structure of the cured polymer was found suitable for electron and epifluorescence microscopy, and it contained an additional component of filler material, which needed to be investigated. The precursors of the polymer could be dissolved in 2-propanol.

Two test models were developed and afterwards applied to different polymers when feasible.

Determination of Structural Integrity and Viability of Encapsulated Endospores in Cured Polymer (Test Model I)

A surface embedding model was newly developed as a method to allow easy localization of intentionally embedded spores in the cured polymer block, and to facilitate the access for microscopic and electron microscopic techniques. Inoculated spores were partially or completely embedded to a depth of several spore diameters. Hence, they were directly accessible for analytical examination without the necessity of sectioning or fractioning to disrupt the polymer. This preparation method allowed application of a range of detection methods as shown in Results Section 2. This test model was only applied to Scotch-Weld 2216 B/A.

Investigation of Uncured Polymer Precursors to Quantitatively Estimate the Worst Case Bioburden (Test Model II)

While Test Model I allowed localization and characterization of experimentally embedded spores in high numbers, any inherent bioburden of the polymers would be too dispersed to allow detection by microscopic methods *in situ*. Chemical or physical disintegration of the polymers would have to be applied to extract intrinsic encapsulated microorganisms from cured polymers. In contrast to the experiments performed on lucite [26], solvent dissolution of the cured spacecraft relevant polymers investigated here, was not feasible. Mechanical degradation of the polymer was considered possible, but exceedingly difficult to control with regard to uniformity of the procedure and mechanical spore disintegration. Hence, no advantage was seen in milling or grinding procedures. As an innovative approach, determination of the bioburden of the uncured polymer precursors was pursued. During the terminal step of polymerization (curing) an increase in polymer bioburden is obviously impossible, while physical and chemical processes during polymerization might even reduce viable microorganisms. Hence, bioburden in uncured polymer was considered the most conservative proxy for encapsulated bioburden in cured polymer. As all uncured polymer precursors were found to be soluble in suitable organic solvents, the test model was applicable to all the spacecraft relevant polymers investigated here.

For all uncured polymers, survival of inoculated spores of the *B. safensis* model organism during a 60 min exposure period was verified. This was performed at high spore concentrations (10^7 cfu/mL) to allow dilution in buffer and to minimize the effect of solvent and polymer residues in the plating assay.

To achieve maximum sensitivity in determination of inherent bioburden, polymer precursors were diluted in solvent as little as possible and pour-plated in agar without transfer to an aqueous diluent. For all investigated polymers germination and colony growth of inoculated spores (*B. safensis* 10^2 cfu/mL) in presence of residual polymer and solvent was verified. This also allowed determination of the recovery efficiency for the model organism for each given material and method. Recovery results were used to specify the amount of un-inoculated material that had to be extracted to generate significant figures for estimation of intrinsic endospore burden per cm^3 of cured polymer.

Application of Test Model I: Detection and Viability Determination of Surface-embedded Spores in Cured Scotch-Weld 2216 B/A

Preliminary experiments had shown that ultra-thin sectioning using a microtome followed by transmission electron microscopy (TEM) was not feasible for the material due to the spongy structure of Scotch-Weld 2216 B/A. Instead, surface-embedding followed by scanning electron microscopy was found to be the method of choice to qualitatively demonstrate the presence and accessibility of embedded spores in Scotch-Weld 2216 B/A. Polymerization of the adhesive Scotch-Weld 2216 B/A on a layer of spores on the inoculated surface of water-soluble poly vinyl alcohol (PVA) yielded a surface with fully embedded, partially embedded and loosely attached spores as shown in **Fig. 1A**. To visualize the effect of encapsulation and polymerization on structure and integrity of *G. stearothermophilus* and *B. safensis* spores, scanning electron microscopy (SEM) in combination with focused ion beam (FIB) electron microscopy were employed.

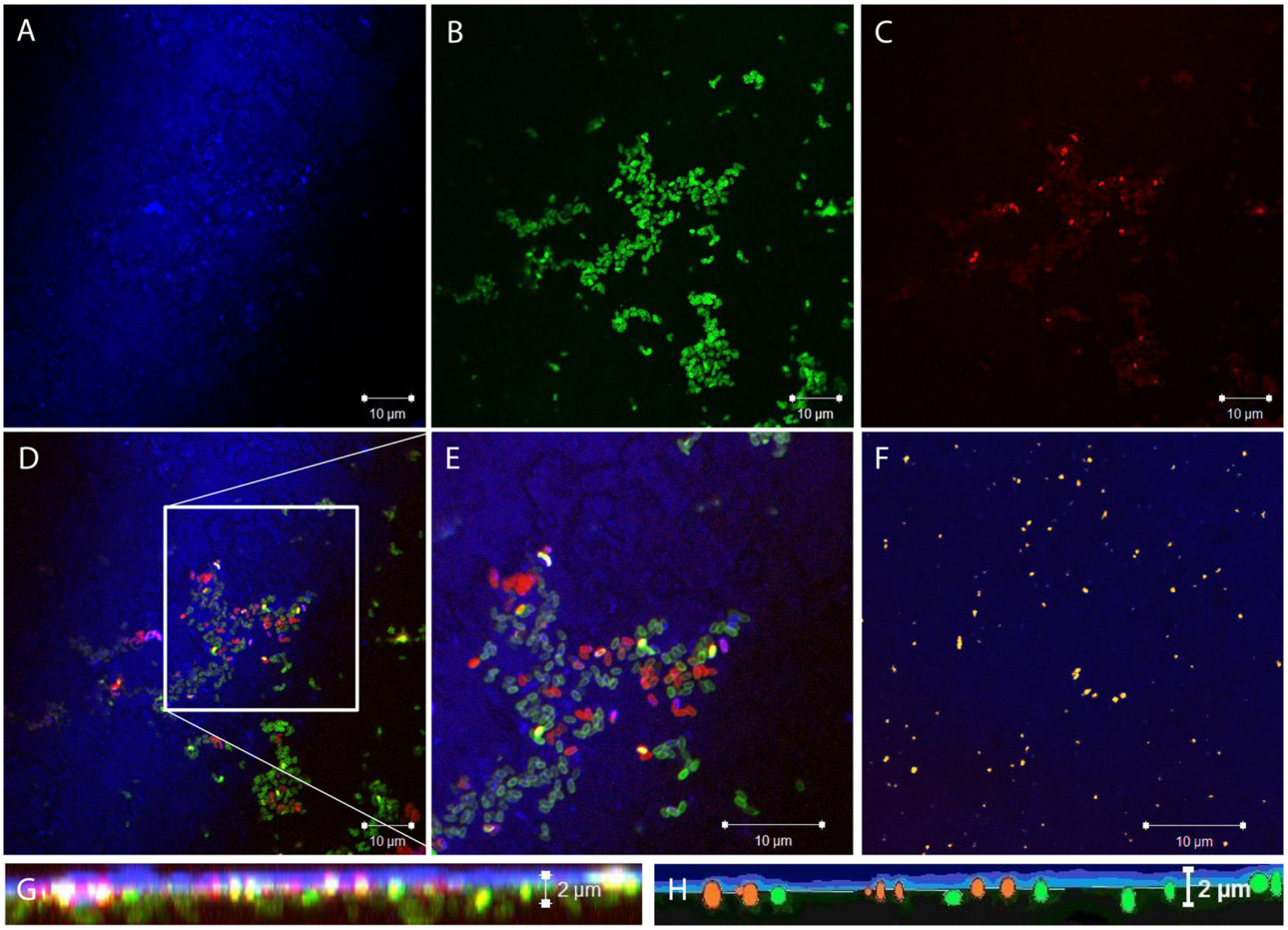

Figure 2. CLSM images of Test Model I. (A) Cy5 channel (blue) to visualize the polymer surface. (B) AF488 channel (green) to distinguish spores from background signals (potential polymer autofluorescence and debris). (C) PMA channel (red) to detect disintegrated spores. (D) overlay of all channels. (E) shows enlarged detail of Fig. 2D. (F) CTC channel (yellow) show spores with metabolic activity in the presence of provided nutrients (different position on Test Model I is shown). (G) z-axe projection from 90° calculated from z-stacks scanned with a 50% overlap of each 0.4 µm section (blue horizontal line indicates the surface of the adhesive). (H) abstracted scheme of Test Model I interpreted from Fig. 2G – intact spores colored in green, disintegrated spores are red-orange, blue glowing line demonstrates polymer surface, gray area represents polymer material. Spores of *B. safensis*, were stained with AF488 prior to embedding (Fig. 2B), but with PMA after embedding (Fig. 2C). The PMA signals were visible in a layer of about 3 µm, whereas the AF488 signal was detectable to a depth of 10 µm (spores were introduced into deeper layers during embedding; PMA could penetrate the polymer to stain spores up to a depth of 3 µm). All images, overlays and projections were achieved with the Zeiss LSM Image Browser Software.
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SEM images of the sample surface confirmed the embedding status (**Fig. 1**). Non-inoculated surfaces were smooth (as seen in in **Fig. 1A** to the right of the embedded spores) without spore structures. Double beam FIB/SEM analysis confirmed the varied depth of surface-embedding as indicated in the SEM analysis. Spores embedded to the depth of several spore diameters could be visualized (**Fig. 1 C**). The double beam FIB/SEM images also showed the presence of filler material in the polymer, which obstructed the clear outlines of the faint shapes of embedded spores.

While the electron-microscopic study allowed visualization of spores and their arrangement with respect to the polymer matrix, for viability determination physiological tests had to be performed. Samples of Scotch-Weld 2216 B/A with surface-embedded *B. safensis* spores were overlaid with nutrient agar after cleaning the surface with 70% ethanol to remove loosely attached spores and inactivate vegetative contaminants. Several colonies developed in the agar, indicating that (partially) embedded spores of *B. safensis* were still cultivable after the curing process.

Application of standard cultivation technique and physiological fluorescent dye staining followed by fluorescence and confocal laser scanning microscopy to surface-embedded samples clearly illustrated the high potential of this preparation method for analysis of polymer-embedded spores.

In order to systematically evaluate the depth to which spores could be introduced into the adhesive by the embedding procedure, spores were stained with AF488 prior to embedding. As a surface marker, Cy5 was employed. Full Cy5 fluorescence was present above the adhesive surface in the liquid layer and disappeared on and below the surface. This allowed exact localization of partially embedded spores in Scotch-Weld 2216 B/A (**Fig. 2A**). Controls without embedded spores and stained with Cy5 and AF488 afterwards revealed only scattered locations of AF488 signals, which could be clearly distinguished from spore shapes and spore label intensity. Staining quality and CLSM channel configurations were adjusted and judged for labelled spores in suspension, labelled spores on polymer surface, as well as embedded and encapsulated labelled spores. A slight decrease in label fluorescence could be detected for embedded and encapsulated spores. AF488-prestained spores were successfully detected to a depth of 9.2 µm by CLSM (**Fig. 2B**). In addition, PMA-staining after the embedding procedure was performed as described in [29] to analyze the effect of polymerization on spore integrity *in situ*. Spores could be stained even after being (partially and fully) embedded in Scotch-Weld 2216 B/A in a layer of approximately 3 µm from the surface (**Fig. 2C–E**). Notably, the process of embedding did not significantly change the percentage of PMA-stainable (disrupted) spores compared to the non-embedded control, indicating that most spores were unaffected by the embedding process (**Table 2**). This was confirmed by successful CTC staining, indicating an active oxidative metabolism of (partially) embedded spores of *B. safensis* after reactivation by nutrient supplement (**Fig. 2F**). Colonies that appeared after covering half-embedded spores with nutrient agar were *B. safensis* colonies, as revealed by *rrnB-16S* (99.9% identity) and *gyrB* gene sequencing (96.48% identity to type strain deposited sequences in GenBank: http://www.ncbi.nlm.nih.gov/genbank/).

Application of Test Model II for Scotch-Weld 2216 B/A: Survival of *B. safensis* Spores in Polymer Precursor

The possible sporicidal effect of the uncured polymer precursors was tested by inoculating Scotch-Weld 2216 B/A as described in Materials and Methods. Cultivable spore recovery directly after inoculation was 59% (**Fig. 3**). The number of retrievable cultivable spores remained stable over the incubation period of 60 min (not shown). An inhibitory effect of the solvent (2-propanol) used for dilution of the material after the incubation period had been experimentally excluded in preliminary experiments.

Application of Test Model II for Scotch-Weld 2216 B/A: Recovery Efficiency of Inoculated Spores from Polymer Precursors in Presence of Organic Solvents

Colony formation of activated spores in the pour-plate medium was only slightly inhibited by the presence of Scotch-Weld 2216 B/A and solvent residues. The recovery efficiency of spores amounted to 89% (**Fig. 3**), which was higher, but in the same order of magnitude as survival in the precursors. This discrepancy was due to the difference in experimental procedure to determine survival and recovery, respectively. In survival determination, a high inoculum was used, which was consecutively diluted before plating, which may have caused some loss of spores. The recovery efficiency (ratio of recovered cfu in nutrient medium in presence and absence of polymers) was used to correct the value for total intrinsic bioburden.

Application of Test Model II for Scotch-Weld 2216 B/A: Encapsulated Viable Intrinsic Bioburden of Uninoculated Polymer Precursors

A recovery efficiency of >10% was arbitrarily specified as acceptance criterion for extraction of natural encapsulated bioburden from polymer precursors because the quantity of the material studied was high (87 g or 66 cm^3 for Scotch-Weld 2216 B/A; **Table 3**; investigated in 80 separate samples). In the total amount of Scotch-Weld 2216 B/A investigated, 0 cfu were found on the agar plates (**Table 3**). Thus, contamination of Scotch-Weld 2216 B/A with spores cultivable under the given conditions was calculated to be below 0.1 cfu/cm^3 (**Table 3**).

Application of Test Model II for Scotch-Weld 2216 B/A: Molecular Determination of Contaminants in Polymer Precursors

Quantitative PCR (qPCR) was applied to estimate the total natural bioburden of the polymer materials, including both dead and potentially viable cells. However, DNA extraction from polymer precursors proved very difficult. Although Scotch-Weld 2216 B/A could be dissolved properly in 2-propanol, remaining residues and clay particles bound biomaterial effectively, so that a separation of polymer and biomolecules (incl. cells and DNA) was impossible. This resulted in a very poor recovery of spore signatures, which were added as positive control (<0.1% DNA extraction efficiency).

Application of Test Model II for Scotch-Weld 2216 B/A: Contribution of the Filler Material Kaolin to the Overall Bioburden Determined by Molecular Methods and Cultivation

Scotch-Weld 2216 B/A contains about 30% (w/w) of kaolin as a mineral filler material (see fiber-like inclusions in **Fig. 1C**). Bioburden of kaolin was determined separately, since dissolution by solvents was impossible. In addition, due to its natural origin, this filler was suspected to be a source of elevated microbial contamination. Kaolin ASP 200, 600 and 900 (3 different grain sizes) were separately tested for microbial contamination by cultivation on three different media as well as by DNA extraction and qPCR. As kaolin could be suspended in water, DNA

Table 2. Influence of embedding in polymerized Scotch-Weld 2216 B/A on *B. safensis* spore integrity.

Sample	PMA positives (%)	Ratio (spore number)	Signal depth AF488 (μm)	Signal depth PMA (μm)
spore suspension (AF488 + PMA)	2.91	37/1272	-	-
Non-embedded control (AF488 + PMA before embedding)	3,74	52/1390	2.8	2.8
Embedded viable spores (AF488 before, PMA after embedding)	3.29	137/4166	2.8	0.8

Only partially-embedded spores were counted (directly on the surface of Scotch-Weld 2216 B/A, treated by ultrasonication; surface location was confirmed by using Cy5 surface marker).

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extraction efficiency was higher than for the final polymeric material Scotch-Weld 2216 B/A. The relevance of kaolin as contamination source for Scotch-Weld 2216 B/A, in consideration of the contribution of the filler to the weight of cured Scotch-Weld 2216 B/A and the density of Scotch-Weld 2216 B/A (1.32 g/cm^3), was determined by the different methods as summarized in **Fig. 4.** Microbial contamination introduced into Scotch-Weld 2216 B/A by kaolin was estimated by qPCR to be in the range of 10^3 (potential vegetative cells or spores)/cm^3. Only a fraction of this number (~1%) could be recovered by cultivation on agar. It should be noted, that the calculated number of microbial contaminants based on molecular methods can only be a rough estimate as the number of *rrnB-16S* gene copies per genome and cell is unknown for unidentified microbes. Furthermore, this method also detects DNA from dead cells, permitting no quantification of viable cells.

Application of Test Model II for other Polymers

In addition to Scotch-Weld 2216 B/A, four other space craft relevant polymers, of different type and composition were also investigated: SG121FD a silicone coating; Solithane 113, a urethane resin; ESP 495 a silicone adhesive; and Dow Corning 93–500 (DC 93–500) a silicone encapsulant (**Table 1**). Bioburden determination of uncured polymer precursors was performed following Test Model II.

Application of test model II for other polymers: survival of *B. safensis* spores in uncured polymer precursors. The possible sporicidal effect of the polymer precursors was tested as described in Materials and Methods. Recovery directly after inoculation was >10% for all tested polymer materials (**Fig. 3**), ranging from 13% in ESP 495 to 60% for DC 93–500. The number of cultivable spores remained stable over the incubation period three of the selected materials, whereas in the uncured silicone coating MAP SG121FD, a reduction of cultivable spores (to 0.03% of the initial inoculum) within 60 min of exposure indicated a pronounced sporicidal effect (**Fig. 5**). An inhibitory effect of the solvent (SG121FD thinner) used for dissolution of MAP SG121FD after the incubation period could be experimentally excluded.

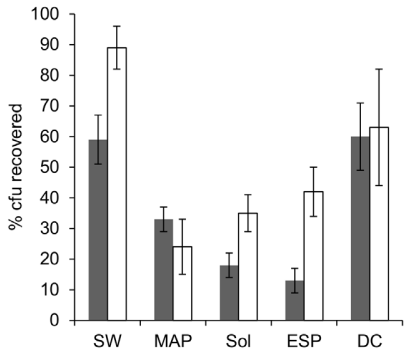

Figure 3. Survival of *B. safensis* spores in uncured polymer precursors (gray bars) relative to the initial inoculum (10^7 spores/mL, dilution in aqueous diluent), and growth of 10^2 cfu of *B. safensis* in the presence of uncured polymer precursors on nutrient agar (white bars) relative to the population of *B. safensis* grown without precursors.

doi:10.1371/journal.pone.0094265.g003

Application of Test Model II for other Polymers: Recovery Efficiency of Spores from Polymer Precursors in Presence of Organic Solvents

Colony numbers of *B. safensis* spores were slightly reduced by the presence of polymers and solvents in the solid growth medium in all cases, but colony counts exceeded 10% (**Fig. 3**), ranging from 24% in MAP SG121FD to 63% in DC 93–500. As noted before, higher recovery efficiencies compared to survival ratios may be due to loss of spores during the serial dilutions performed for survival determination. In general, survival and recovery was in the same order of magnitude.

Application of Test Model II for other Polymers: Encapsulated Cultivable Bioburden of Uninoculated Polymer Precursors

The number of colonies recovered from the unspiked polymer precursors by solvent dilution and incubation in solid nutrient medium was very low in all cases. In 87 g (81 cm^3) Solithane 113, 1 cfu was recovered, giving a corrected bioburden of 0.1 cfu/cm^3. The detected colony consisted of gram-positive, rod-shaped cells with visible endospores. One colony was also recovered from 64 g (59 cm^3) DC 93–500. However, as the isolate did not survive heat shock treatment when suspended in solvent, it was very likely a secondary contamination of vegetative organisms. Bioburden of DC 93–500 was determined to be below 0.1 cfu/cm^3. Investigation of 48 g (45 cm^3) ESP 495 yielded 2 cfu of spore-forming organisms, one of which appeared under elevated temperature (55–60°C) incubation. Although 4 more colonies were recovered, those consisted of vegetative organisms, sensitive to the extraction procedure and were considered secondary contaminations. Thus, the corrected intrinsic bioburden of ESP 495 amounted to 0.3 cfu/cm^3. In MAP SG121FD coating, results varied for the two analyzed batches. 5 cfu were recovered after incubation under mesophilic conditions in the first batch of SG121FD (18 g or 13 cm^3 investigated). Gram-staining of these colonies showed gram-positive, rod-shaped cells, indicating spore-forming organisms. No colony was detected in the second batch of SG121FD, of which a higher amount was analyzed (71 g or 50 cm^3). It should be taken into consideration that cultivability of *B. safensis* spores

Table 3. Contamination of the polymer materials with bacterial endospores determined by dilution in solvent and cultivation (corrected values calculated with recovery efficiency).

Material	Amount of material investigated (cm^3)	Total spore-forming colonies detected (cfu)	Encapsulated bioburden (cfu/cm^3), corrected
Scotch-Weld 2216 B/A	66	0	<0.1
MAP SG121FD (batch 1/batch 2)	13/50	5/0	2.4/<0.3
Solithane 113	81	1	0.1
ESP 495	45	2	0.3
DC 93–500	59	0	<0.1

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was rapidly reduced within 60 min of exposure in SG121FD (**Fig. 5**). The spore-forming organisms, which were detected in one of the batches of SG121FD could have been more resistant to the inhibitory effects of the silicone coating than *B. safensis*.

Application of Test Model II for other Polymers: Molecular Determination of Contaminants in Uninoculated Polymer Precursors

Solithane 113 was selected in addition to Scotch-Weld 2216 B/A to attempt the application of molecular methods. When attempting to extract DNA from Solithane 113, similar obstacles arose as for Scotch-Weld 2216 B/A. Although diluted in ethanol and using an adapted DNA extraction protocol, uncharacterized polymer residues masked targeted biomolecules for subsequent molecular analyses. 2×10^6 spores were added as a positive control, resulting in the detection of approx. 10^4 *rrnB-16S* gene copies via qPCR (less than 0.5% extraction efficiency; in case the average spore contains more than a single gene copy, the extraction efficiency would be even lower, as expected. In addition, polymer components in the DNA suspension caused interference with the qPCR signal, which had to be adjusted by application of a correction factor. Nevertheless, Solithane 113 samples revealed a higher contamination with *rrnB-16S* genes than positive controls (**Fig. 6**). Since the recovery efficiency for the total natural bioburden from Solithane 113 is unknown, the true bioburden cannot be estimated. However, a minimum contamination of approx. 4×10^3 gene copies per 5 g material (500/g or 535/cm^3) can be envisaged.

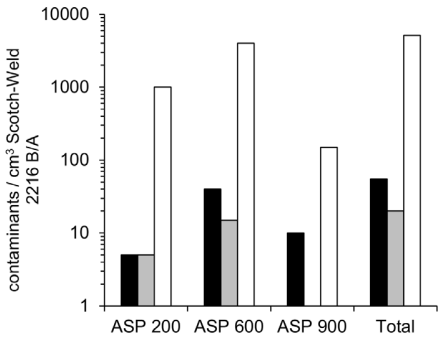

Figure 4. Microbial contamination per cm^3 Scotch-Weld 2216 B/A contributed by filler material kaolin (3 grain sizes ASP 200, 600 and 900), as determined by cultivation (cfu = without heat shock, black bars; cultivable spores = heat-shock-survivors, gray bars) and by qPCR (total contaminants (*rrnB-16S* gene copies, white bars). Standard deviations are not shown as the data is only meant to give a broad orientation. For a statistically sound analysis a much higher sample number would need to be investigated.
doi:10.1371/journal.pone.0094265.g004

Discussion

Planetary protection ensures the validity of future life detection missions on foreign celestial bodies by biological control of spacecraft and its assembling environment in clean room facilities [42]. Since the Viking mission era, spacecraft surface associated bacterial spore burden has been assessed by standard cultivation protocols [43,44,45,46]. Recent approaches were based again on cultivation-level [47], or applied new promising molecular methods, since cultivation is limited to standard laboratory conditions and bears the risk to ignore the majority of microorganisms in a sample [48,7,49,50]. The knowledge about polymer encapsulated bioburden, however, remains sparse, most likely due to difficulty to retrieve the organisms from cured polymers. Milling of cured polymers was considered impractical as it involves mechanical shear forces and local heating, which are very difficult to control and their effect on encapsulated cells or spores is unknown [51,52]. Comparable hurdles have been described by [14] and [26] during recovery and detection of encapsulated bioburden in silicone or Plexiglas and had to be overcome by adapted extraction and permeabilization protocols. While the polymers used in the aforementioned studies were selected specifically to study encapsulation models, the aim of the present study was to find test models that can be used to characterize the intrinsic bioburden of spacecraft-relevant polymers. The test models developed for Scotch-Weld 2216 B/A, and applied to ESP 495, DC 93–500, MAP SG121FD and Solithane 113 can be used for varied test strategies to characterize and

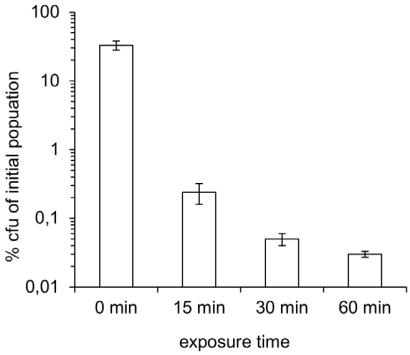

Figure 5. Survival of *B. safensis* spores in uncured MAP SG121FD during 0–60 min of incubation.
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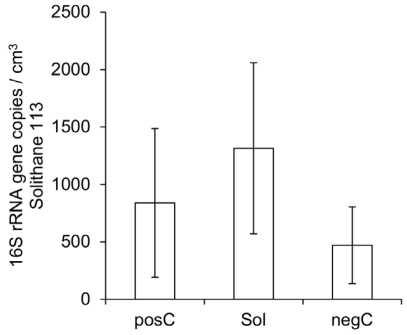

Figure 6. Mean molecular biocontamination of Solithane 113 determined by qPCR.
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estimate the intrinsic bioburden of polymers. The models lend themselves to investigate not only the most frequently used polymers for spacecraft but also other polymers used in food and pharmaceutical industry or medical devices.

We first developed a surface-embedding model that allowed easy access to the inoculated spores for assessment of the effect of polymerization on encapsulated *B. safensis* spores in Scotch-Weld 2216 B/A as an exemplary polymer. Without any disruptive extraction from the polymer, embedding effects could be studied with a minimum of preparation artifacts. Fluorescently labelled spores, which could be detected to a depth of 3 μm, showed spore coat integrity and respiratory metabolism in presence of provided nutrients and some of them were able to germinate and form colonies. Thus, it cannot be assumed that the polymerization process is damaging to spores, although this result is expected to vary with different polymeric materials as well as with spore structure. For materials that lend themselves to application of the described or similar methods, our developed encapsulation model in combination with molecular staining techniques can be a valuable tool to assess how the germination ability of spores is affected by polymerization and gives additional information about embedded spores to the approach with Alexa-FISH presented by Mohapatra and La Duc [26].

Although the model presented by Mohapatra and LaDuc was pioneering work and yielded interesting data on spiked spores, it could not be applied to quantitatively estimate the intrinsic bioburden of polymers. The intrinsic bioburden of polymers is expected to vary for a number of reasons: i) they are composed of widely different materials, ii) some include materials of natural origin (e.g. fillers), iii) manufacturing conditions for the polymers are diverse and iv) uncured materials may have different effects on microorganisms. For estimation of intrinsic bioburden in a broad range of polymers a method for lot-specific quantitative estimation of the intrinsic bioburden was sought. We developed a test model that combines cultivation-based and molecular methods for quantitative bioburden determination. Since biocompatible disruption or dissolution of the cured polymers was found to be impossible in this study, uncured polymer precursors were diluted in solvents before polymerization. This was considered the most conservative and also the least sporicidal approach. Inoculated endospores (shown to be insensitive to the employed diluents) could be recovered with reasonable effectiveness. Hence, the strategy applied herein was shown to be applicable to determine the maximum number of cultivable intrinsic spores in a wide range of polymers.

A low contamination with encapsulated cultivable endospores was found for all investigated polymer materials, in the range of $<$ 0.1 spores/cm^3 to a maximum of 2.4 spores/cm^3 in one batch of MAP SG121FD. SG121FD was the only tested material, for which a sporicidal effect on inoculated model spores was observed over incubation periods of $\leq$60 min. Thus, the risk of germinable spore contamination for this material was considered to be low.

The bioburden of polymer precursors is specific for each material and can vary from batch to batch (as shown for MAP SG121FD). For practical application a number of batches would have to be tested, and any change in the manufacturer or the manufacturing process of the polymer material would necessitate re-evaluation of its bioburden.

As for previous attempts of bioburden evaluation [43,44,45], the limitation of this method was the use of cultivation-based detection methods, which are known to detect only a fraction of the total number of viable organisms present in a sample of natural origin [53,54,55]. However, if the recovered spores are unable to germinate under optimal conditions, the probability for germination and propagation on a possibly hostile planetary surface might be considered minute, even if phenomena like superdormant spores are regarded [56].

Nevertheless, for planetary protection purposes, dead and not-yet-culturable organisms need to be considered as well, since they might interfere with life or biomarker detection methods. Therefore, determinations of the molecular bioburden were considered an important complement to cultivation-based assays. However, DNA extraction from the polymer precursors of Scotch-Weld 2216 B/A proved difficult, because DNA was bound by the polymer particles diluted in solvent as described to be the case for silicones, too [14]. Hence, promising high throughput omics technologies (e.g. transcriptomics and proteomics) could not be applied beyond our qPCR and microscopy based approaches. Due to the low extraction efficiency, quantitative data could not be obtained for Scotch-Weld 2216 B/A, but for its filler kaolin, which amounted to $\sim$10^3 cells/cm^3 (estimation from *rrnB-16S* gene copies determined via qPCR). This amount exceeded by far the number of microorganisms that could be detected by cultivation in the same material. However, a general conclusion for bioburden of this filler is difficult to draw, since kaolin is a natural resource originating in clay mines, which are exposed to various environmental influences, and harbor a remarkable diversity of extremophiles like iron oxidizing and reducing bacteria (dark iron oxides and sulfides are major discoloring impurities of white kaolin [57]), and acidophilic archaea and bacteria (sulfidic mine waters [58]). Additionally, mining and processing of kaolin is not performed under sterile conditions and may vary for different manufacturers. Nevertheless, this result was in accordance with our expectations, as molecular methods include DNA from dead organisms, as well as from viable and viable-but-nonculturable organisms. The high numbers of potentially viable microbial contaminants (also found in previous studies by other authors, e.g. [59], indicate that fillers of polymers should not be dismissed in studies of encapsulated bioburden.

Recommendations for Future Studies

We recommend the application of the presented methods to other materials for which an evaluation of encapsulated bioburden is important. Such materials may be other adhesives, coatings (silicone) and bulk polymers (polyurethane, silicone and polyimide), honeycomb panels, heat shields (Norcoat Liege cork) and components like wires and connectors [60]. Since these materials are quite diverse, in each case a risk analysis is recommended to decide, if viable bioburden is expected to be present at all. For composite materials like wires and connectors it is important to investigate whether bioburden resides in the polymeric insulation

materials, in between the insulating layers, or on the wires themselves. The testing strategy will have to be adjusted accordingly, and a special set of methods for evaluating its bioburden will have to be posed.

The method of choice for components where dissolvable precursors are available is Test Model II, resulting in the most conservative evaluation for embedded spores. A suitable surface embedding model where added microorganisms are easy to detect and analyse in various depths of embedding as presented in our Test Model I could be helpful for evaluating polymerization effects on the viability of other microbes.

Presented physiological markers with fluorescent activity are quick and applicable given that the material itself does not show severe auto-fluorescence. If the material has to be considered a potential contamination risk and especially if auto-fluorescence is preexisting [26], a separate analysis (e.g. qPCR) of certain components as performed for the kaolin filler might be appropriate. As revealed by our study, separate sterilization of such natural components would be a good alternative in order to reduce the viable encapsulated bioburden. In addition, it might also be important to phylogenetically, metabolically and physiologically characterize the detected bioburden to estimate its risk for planetary protection.

Conclusion

With polymeric components of spacecraft sent from earth to other celestial bodies, but also with the ubiquitous use of polymer materials in food and pharmaceutical industry, the question of their possible internal bioburden has been raised. Here, we have investigated methods that can be applied to estimate the number of persistent microorganisms encapsulated in different polymeric materials. The methods, which were presented as a composite strategy by use of viability staining of embedded microorganisms, classic cultivation procedures, and molecular detection techniques to evaluate encapsulated bioburden of spacecraft model hardware, are not only of interest for the research field of planetary protection. For food [61] and pharmaceutical industry [62,63,64] and clinical equipment [65], an evaluation of cleanliness and sterility is also of great importance, since these fields are confronted by new threats like multi-resistant bacteria and pathogens [66]. Bioburden embedded in polymeric materials is of interest wherever plastic materials are used. Survival of bacterial endospores embedded in such materials is of significance when the huge amount of plastic materials is considered, that is rotting in dumps or littering the surface of the earth and the oceans [67]. These materials do not only leach chemicals but may also harbor microorganisms that get released and proliferate.

Our protocols proved feasible for five typical spacecraft polymers, and we can state that the overall cultivable endospore burden inside the analyzed polymers was low with <0.5 spores/cm^3 for most of the investigated materials. Furthermore, beyond cultivation and molecular-based methods, this study shows the potential of physiological staining methods like AF488, PMA and CTC to evaluate embedded spores in polymers. The combination of these dyes demonstrated for the first time the capability of spores to withstand mechanical forces during embedding in and curing of a polymer.

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Author Contributions

Conceived and designed the experiments: CM KH. Performed the experiments: A. Bauermeister AM AA A. Böker NF CW AJP. Analyzed the data: A. Böker AM AJP CM KH. Contributed reagents/materials/analysis tools: A. Böker. Wrote the paper: A. Böker AM CM KH.

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Quo vadis? Microbial profiling revealed strong effects of cleanroom maintenance and routes of contamination in indoor environments

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Space agencies maintain highly controlled cleanrooms to ensure the demands of planetary protection. To study potential effects of microbiome control, we analyzed microbial communities in two particulate-controlled cleanrooms (ISO 5 and ISO 8) and two vicinal uncontrolled areas (office, changing room) by cultivation and 16S rRNA gene amplicon analysis (cloning, pyrotagsequencing, and PhyloChip G3 analysis). Maintenance procedures affected the microbiome on total abundance and microbial community structure concerning richness, diversity and relative abundance of certain taxa. Cleanroom areas were found to be mainly predominated by potentially human-associated bacteria; archaeal signatures were detected in every area. Results indicate that microorganisms were mainly spread from the changing room (68%) into the cleanrooms, potentially carried along with human activity. The numbers of colony forming units were reduced by up to ~400 fold from the uncontrolled areas towards the ISO 5 cleanroom, accompanied with a reduction of the living portion of microorganisms from 45% (changing area) to 1% of total 16S rRNA gene signatures as revealed via propidium monoazide treatment of the samples. Our results demonstrate the strong effects of cleanroom maintenance on microbial communities in indoor environments and can be used to improve the design and operation of biologically controlled cleanrooms.

The vast majority of microorganisms is known to play essential roles in natural ecosystem or eukaryote functioning[1]. However, the indoor microbiome is only at the beginning of being explored and could have severe impact on human health, well-being or living comfort[2,3]. Next generation sequencing and OMICS-technologies have tremendously contributed to the census of microbial diversity and enabled global projects analyzing terrestrial, marine, and human microbiomes[1,4,5]. These techniques opened up also new possibilities to study indoor microbiomes, which are an important component of everyday human health[6–8]. In general, uncontrolled indoor microbial communities are characterized by a high prokaryotic diversity and are comprised of diverse bacterial and archaeal phyla[7–11]. The microorganisms originate mainly from the human skin or from outside air and soil, and have even been known to include extremophiles[12]. In addition, the plant microbiome was suggested as important source for indoor microbiomes[13]. Although numerous developments and improvements have been reported during the last decade, the proper monitoring and control of microbial contamination remains one of the biggest challenges in pharmaceutical quality control, food industry, agriculture or maintenance of health-care associated buildings, including intensive care units[14–16].

Another important research area dealing with indoor microbiomes is planetary protection, which aims to prevent biological contamination of both the target celestial body and the Earth[17]. Space missions that are intended to land on extraterrestrial bodies of elevated interest (in chemical and biological evolution and signifi-

cant contamination risk) are subject to COSPAR (Committee on Space Research) regulations, which allow only extremely low levels of biological contamination. However, all space agencies that involve in life-detection and sample return missions should consider to catalogue microbial inventory associated with spacecraft using state-of-the art molecular techniques that enable not to compromise the science of such missions. At present, all space agencies enumerate heat-shock resistant microorganisms as a proxy for the general biological cleanliness of spacecraft surfaces that are bound to Mars (COSPAR planetary protection policy; ECSS (European Cooperation for Space Standardization)-Q-ST-70-55C[18]).

In order to avoid contaminants as much as possible, spacecraft are constructed in highly controlled cleanrooms that follow strict ISO and ECSS classifications (ISO 14644; ECSS-Q-ST-70-58C, http://esmat.esa.int/ecss-q-st-70-58c.pdf and https://www.iso.org/obp/ui/#iso:std:iso:14644:-1:ed-1:v1:en). Cleanrooms for spacecraft assembly were the first indoor environments, which were extensively studied with respect to their microbiome[10,19–23]. As expected, the detected microbial diversity and abundance strongly correlated with the applied sampling and detection methods, and during the last years a vast variety of bacterial contaminants was revealed[18,24]. The aforementioned studies gave rise that cleanroom microbiomes are mainly composed of human-associated microbes and hardy survival specialists and spore-forming bacteria as they can tolerate harsh cleanroom conditions[24]. However, cultivation assays that even included media for specialized microbes like anaerobic broths need to be complemented with molecular assays due to the vast majority of uncultivated microorganisms in general[24]. These molecular methods enabled the detection of archaea as a low but constant contamination in cleanrooms, and their presence was linked to human activity; the human body and in particular the skin was shown to function as a carrier of a variety of archaea and is therefore responsible for the transfer of these organisms into cleanrooms[25]. In contrast to general office or other indoor areas, controlled indoor environments, such as cleanrooms, represent an extraordinary, extreme habitat for microorganisms: the exchange with the outer environment is limited as much as possible, the air is constantly filtered, and particles are vastly reduced and frequent cleaning and/or disinfection of surfaces is performed. To date none of the previous research activities have focused on the real effect of cleanroom maintenance procedures on the diversity and abundance of microorganisms and compared the microbiome to typical indoor environments, such as an office facility.

To overcome this gap of knowledge, we have analyzed a cleanroom complex operated by Airbus Defence and Space GmbH in Friedrichshafen, Germany. The controlled environments at this complex are not monitored for biological contamination but provide an excellent research object in order to determine the baseline contamination level and possible contamination routes. The Airbus Defence and Space complex harbors uncontrolled rooms: an office (check-out room, CO), a changing room (UR) and two controlled cleanrooms of different ISO certification in very close vicinity (CR8, CR5; Fig. 1). We used four different methods, which were cultivation, classical 16S rRNA gene cloning, 454 pyrotagsequencing and PhyloChip G3™ technology, in order to analyze the microbial diversity and abundance of these four separated modules at a cleanroom facility. In addition, we performed network analyses to visualize the microbial contamination tracks within the entire facility.

Results

Abundance of microorganisms decreased from uncontrolled to controlled areas. In general, the distribution of cultivable microbes on facility floors was very heterogeneous. Wipe samples taken in one room revealed highly variable colony counts of up to three orders of magnitude. However, technical replicates from one wipe were comparably low in variation with respect to obtained colony counts (representatively, original data for oligotrophs and alkaliphiles are given in Table S1). As shown in Table 1, the changing room (UR) revealed the highest colony counts of cultivable oligotrophs (17.2×10^3 colony forming units (CFU) per m^2), alkaliphiles (1.9×10^3 CFU per m^2) and anaerobes (44.4×10^3 CFU per m^2), whereas the lowest numbers of cultivable microorganisms were detected in the CR5 cleanroom (0.4×10^3, 0, 0.1×10^3 CFU per m^2, respectively). This corresponds to an at least 40-fold reduction of CFUs towards CR5. Bioburden determination according to ESA standard protocols revealed the highest number of CFU in CO samples (heat-shock resistant microbes: 0.2×10^3 CFU per m^2) and UR (without heat-shock).

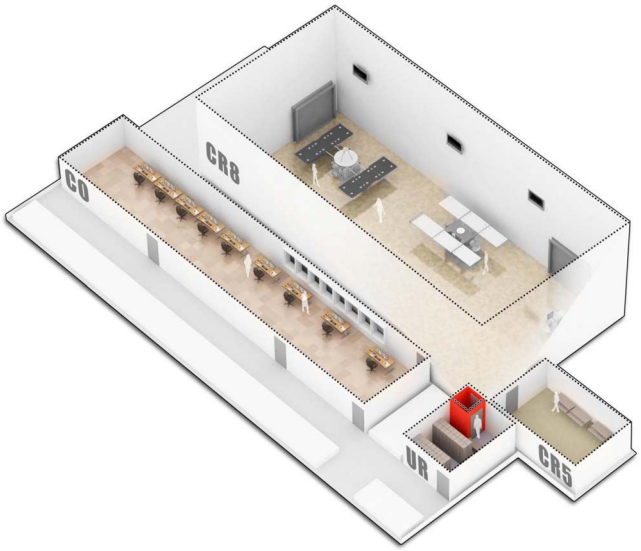

Figure 1 | Illustration of the integration center at Airbus Defence and Space GmbH in Friedrichshafen, Germany. Sampled rooms were designated as follows: UR – changing room, CO – checkout room, CR8 – ISO 8 cleanroom, CR5 – ISO 5 cleanroom. Proportions reflect actual dimensions. Interieur decorations were abstracted and do not mirror real arrangement.

These cultivation-based observations were confirmed by qPCR analyses of wipe samples, which revealed the highest contamination in the check-out as well as the changing room (both approx. 3×10^7 gene copies per m^2), corresponding to an estimated microbial contamination of about 7×10^6 cells per m^2 (in average 4.2 16S rRNA gene copies per bacterial genome[26]). The two cleanrooms revealed an order of magnitude lower gene copy numbers (Table 1). When samples were pre-treated with PMA to mask free DNA (i.e. DNA not enclosed in an intact cell membrane), detected copy numbers per m^2 were even lower: 5.5×10^4 (CR5), 2.6×10^5 (CR8), 2.0×10^6 (CO) and 1.3×10^7 (UR; Table 1). The changing room (UR) thus revealed the highest portion of intact cells (45% of 16S rRNA gene copies).

Cultivation approach revealed the omnipresence of *Staphylococcus*, *Bacillus* and *Micrococcus* in all areas and a great diversity overlap of changing room with cleanroom areas. Cultivation on alternative media (for oligotrophic, anaerobic, and alkalitolerant microorganisms) revealed the presence of facultatively oligotrophic and facultatively anaerobic microorganisms in all rooms. Alkalitolerant microorganisms were not detected in CR8 and in very low abundance in CR5 (Table 1). The relative distribution of identified isolates across the facility rooms is depicted in Fig. 2. A complete list of all isolates is given in Table S2. The most prevalent microbes were staphylococci and *Microbacterium*, whereas *Staphylococcus* representatives were retrieved from each location and *Microbacterium* from CR8 and UR. The overwhelming majority of the isolates obtained from CR5 were identified as representatives of the genus

Table 1 | Microbial abundance and diversity, determined by cultivation- and molecular-based methods

		location			
Cultivation dependent (abundance)		CO	UR	CR8	CR5
CFU per m^2	Oligotrophs	2.3×10^3 ($0.6–4.0 \times 10^3$)	17.2×10^3 ($10.4–23.9 \times 10^3$)	15.7×10^3 ($0.1–31.3 \times 10^3$)	0.4×10^3 ($0–0.7 \times 10^3$)
	Alkaliphiles	0.6×10^3 ($0–1.2 \times 10^3$)	1.9×10^3 ($0.7–3.0 \times 10^3$)	BDL	0* ($0–0.01 \times 10^3$)
	Anaerobes	9.9×10^3 ($6.2–13.6 \times 10^3$)	44.4×10^3	4.1×10^3 ($3.3–4.9 \times 10^3$)	0.1×10^3 ($0–0.3 \times 10^3$)
	Spore Bioburden (heat-shock: 80°C; 15 min)	0.2×10^3 ($0–0.3 \times 10^3$)	0.1×10^3 ($0–0.2 \times 10^3$)	0.08×10^3 ($0–0.02 \times 10^3$)	0.01×10^3 ($0–0.01 \times 10^3$)
	Bioburden (cultivable counts without heat-shock)	3.2×10^3 ($0.8–4.5 \times 10^3$)	TNTC (4.3×10^3- TNTC)	0.7×10^3 ($0.3–1.3 \times 10^3$)	0.3×10^3 ($0.2–0.6 \times 10^3$)
Cultivation independent (abundance)		CO	UR	CR8	CR5
Total bacterial population (16S rRNA gene copies)	qPCR	3.1×10^7	2.9×10^7	0.3×10^7	0.6×10^7
	PMA-qPCR	0.2×10^7	1.3×10^7	0.03×10^7	0.006×10^7
Cultivation independent (diversity)		CO	UR	CR8	CR5
Bacterial diversity (Shannon-Wiener index)	cloning	2.39	2.21	2.15	2.19
	Cloning PMA	1.70	1.60		
	Pyrotagsequencing	6.04	4.76	5.72	5.43
	PhyloChip G3	6.83	6.22	6.89	6.44
	PhyloChip G3 PMA	5.24	0.69	4.09	2.48

*4,4.
TNTC: Too Numerous To Count.
BDL: below detection limit

Staphylococcus (*S. caprae, S. capitis, S. lugdunensis, S. pettenkoferi*), whereas most of the colonies were observed under nutrient-reduced conditions (oligotrophic; Fig. 2). *Erwinia* and *Cellulomonas* were only retrieved from cleanroom samples (CR8). The changing room shared four genera (*Acinetobacter, Propionibacterium, Rhodococcus, Microbacterium*) with the cleanroom environment. Except the three omnipresent cultivated genera *Staphylococcus*, *Bacillus* and *Micrococcus* no additional overlap was found for check-out room and cleanrooms, (Fig. 2). Overall, most CFU were obtained from phyla *Firmicutes* (98), *Actinobacteria* (49) and *Gammaproteobacteria* (10). Only two colonies of a *Bacteroidetes*-representative were obtained (*Chryseobacterium*; UR only).

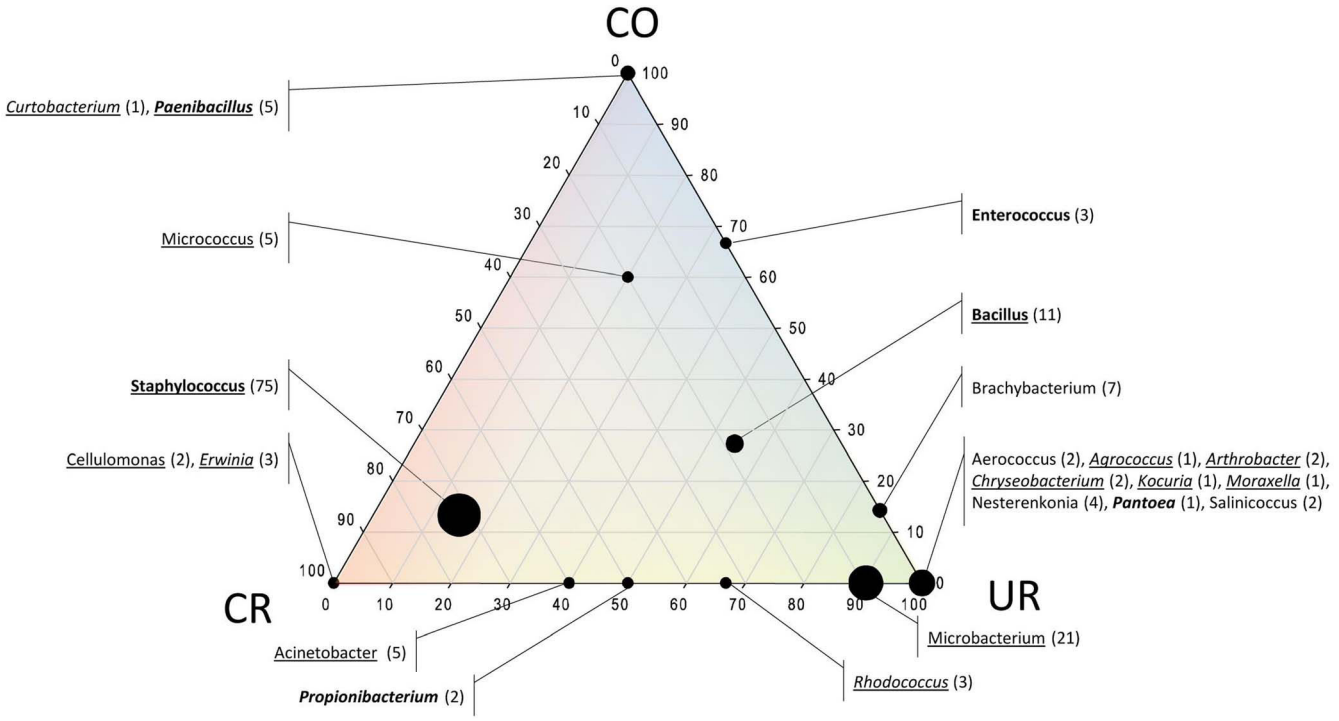

Figure 2 | Ternary plot of isolates (genera) with respect to the sample origin (the two cleanrooms CR5 and CR8 were summarized: CR). Axes reflect the percentage of isolates detected in each location. Isolates obtained under oligotrophic conditions are underlined, isolates obtained under anaerobic conditions are printed bold, isolates obtained under alkaline conditions are printed non-italics. In brackets: number of retrieved colonies.

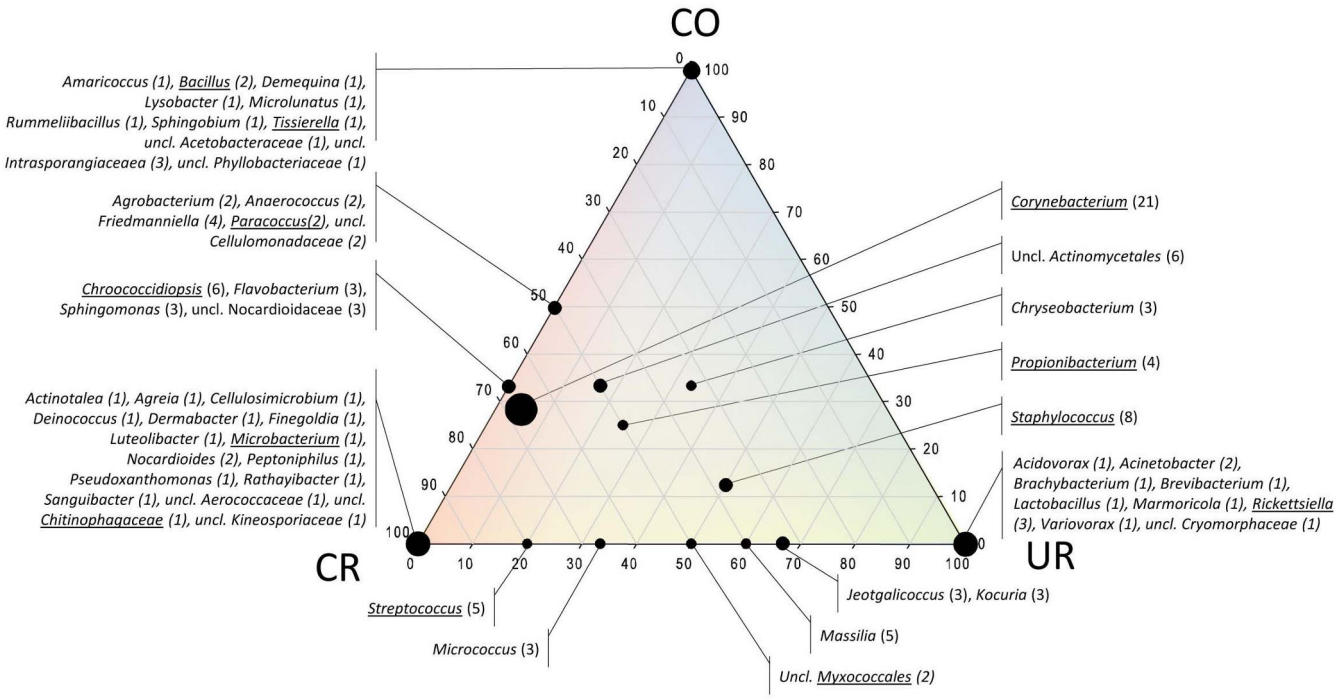

Figure 3 | Ternary plot of detected cOTUs (bacterial 16S rRNA gene cloning) with respect to the sample origin (the two cleanrooms CR5 and CR8 were summarized: CR). Axes reflect the percentage of OTUs detected in each location; OTUs that could not be attributed to an order, family or genus were not considered. Size of dots reflects no. of detected OTUs summarized in one dot. Underlined genera were also detected when samples were treated with PMA (=intact cells). Uncl.: unclassified.

PMA-16S rRNA gene cloning identified *Corynebacterium* and *Staphylococcus* as intact contaminants in cleanroom areas. An overview of all samples processed, no. of clones analyzed and results from grouping into (cloned) operational taxonomic units (cOTUs) and coverage is given in Table S3. In total, 52 sequences (out of 257) were identified to be chimeric and therefore excluded from the subsequent analyses. Chimeric sequences were only detected in samples not treated with PMA. A detailed table of all analyzed recombinant sequences, their abundance and classification is given in Table S4. Fig. 3 displays the microbial diversity detected with respect to their presence in samples from the different facility areas. Sequences of *Corynebacterium*, *Staphylococcus*, *Propionibacterium*, *Chryseobacterium* and members of the order *Actinomycetales* were detected in each of the locations. The most restricted cleanroom

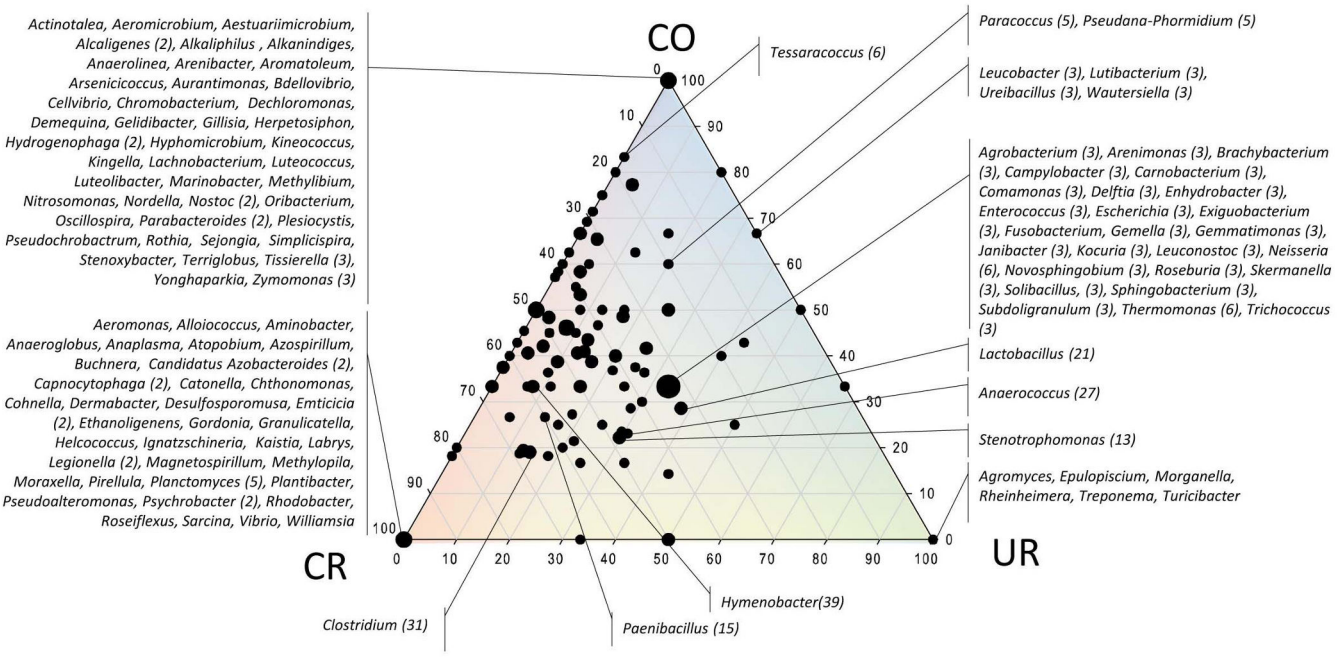

Figure 4 | Ternary plots of detected pOTUs (454 pyrotag sequencing) with respect to the sample origin (the two cleanrooms CR5 and CR8 were summarized: CR). Axes reflect the percentage of OTUs detected in each location; OTUs that could not be attributed to a order, family or genus were not considered. Size of dots reflects no. of detected OTUs summarized in one dot (no. given in brackets if different from 1).

(CR5) exclusively revealed the presence of *Aerococcaceae, Nostocaceae* and *Deinococcus* signatures.

PMA-treatment of samples allowed the detection of signatures from intact cells of *Corynebacterium* (UR, CR5), *Microbacterium, Propionibacterium, Streptococcus, Brevundimonas, Chroococcidiopsis, Ralstonia, Rickettsiella* (UR), *Propionicimonas, Paracoccus, Chitinophagaceae, Bacillus, Myxococcales, Tissierella* (CO) and *Staphylococcus* (UR, CR8). With the exception of omnipresent microorganisms (see above), no overlap occurred between sample diversity obtained from UR samples (changing room) and check-out facility (Fig. 3).

Alpha diversity analysis of pyrotagsequencing suggested an opposed distribution of *Proteobacteria* and *Firmicutes* signatures in controlled and uncontrolled areas. On average, 1863 bacterial 16S rRNA gene sequences were obtained from each sample. Normalized data revealed the highest microbial diversity (see Table 1) in the checkout (6.0 H′) and the lowest in the changing room (4.76 H′). Nine bacterial phyla were detected after setting a threshold of 1% relative sequence abundance, whereas *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, and in particular *Firmicutes* and *Proteobacteria* revealed the highest sequence abundance (see Table S5 and Fig. 4). Bacterial 16S rRNA genes belonging to the phylum *Actinobacteria* were most relative abundant in the checkout room and appeared lower in all other samples (10%). *Bacteriodetes* sequences could be detected in all rooms with a constant relative abundance, with *Wautersiella falsenii* signatures predominating in changing room (UR) amplicons. Sequences of the genus *Tessaracoccus* (*Actinobacteria*) were exclusively found in the checkout (CO). A detailed look at the phylum *Proteobacteria* revealed *Rhodocyclaceae* sequences as most abundant in CO samples, sequences affiliated to the genera *Stenotrophomonas* and *Comamonas* as the most abundant in UR, and *Paracoccus yeei* as the most abundant proteobacterial signature in CR8. Within the *Firmicutes*, 16S rRNA gene signatures of *Aerococcaceae* were predominant in CR5. On genus level, *Anaerococcus* sequences dominated in CR5 and *Paenibacillus* sequences in CR8. Signatures of the species *Finegoldia magna* could be detected in the changing and both cleanrooms with the highest abundance in CR5, whereas *Lactobacillales*-sequences (including *Lactobacillus* and *Lactococcus*) were predominant amongst *Firmicutes* signatures in UR and CO. Noteworthy, the relative abundance of *Firmicutes* sequences increased towards the cleaner areas (CR8, CR5; rel. abundance: 17–45%), whereas proteobacterial pyrosequenced operational taxonomic units (pOTUs) decreased (37–23%) compared to CO

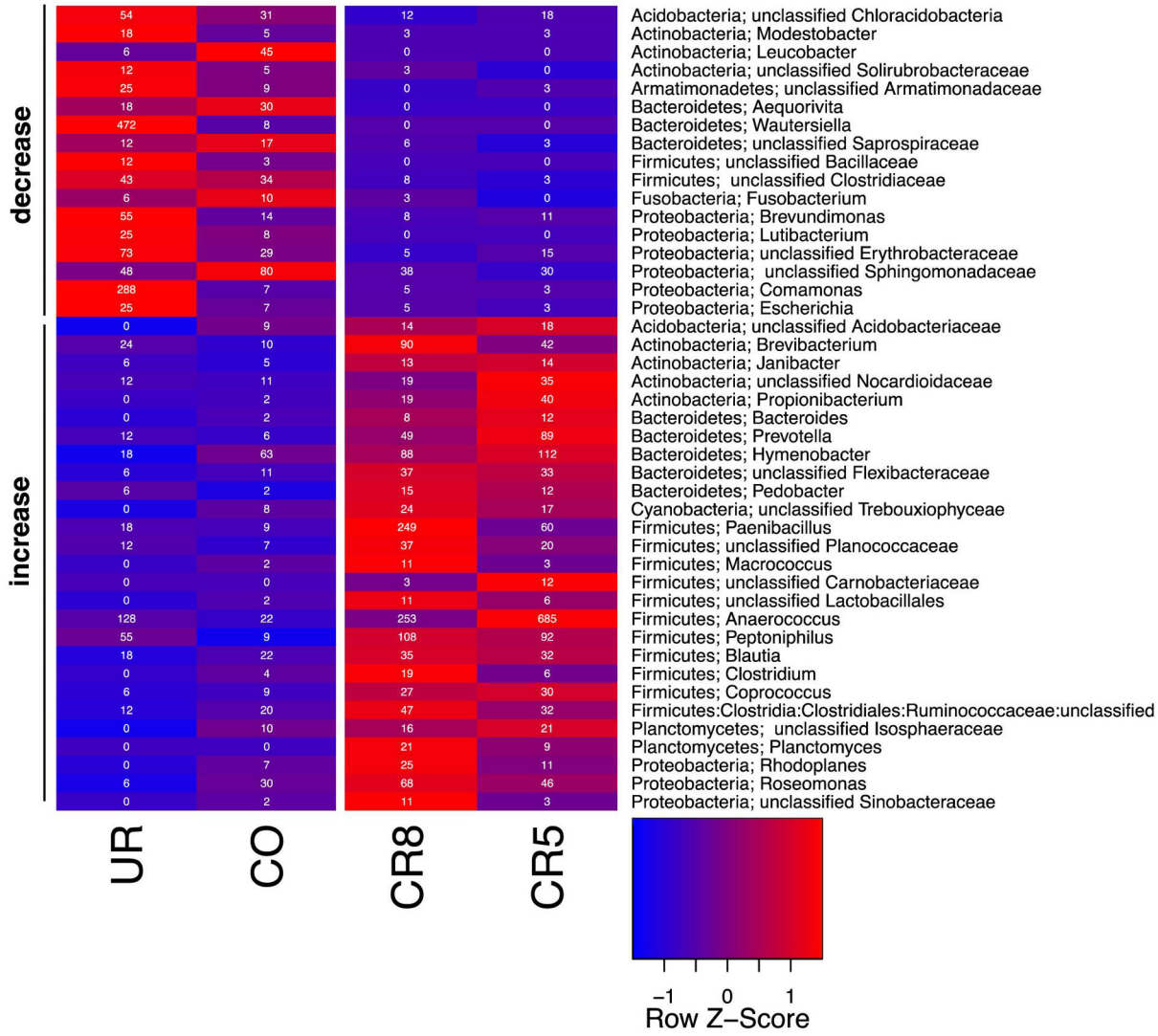

Figure 5 | Heatmap based on 454 pyrotagsequencing data of aggregated read counts at genus level (reads were sum-normalized prior to aggregation). Displayed are genera that showed an at least 25% increase or decrease in both cleanroom samples compared to non-cleanroom samples and had a minimum number of reads of at least 10. Numbers in the cells give number of reads. For the color gradient, read scores were normalized for each genus and are presented as Z-scores.

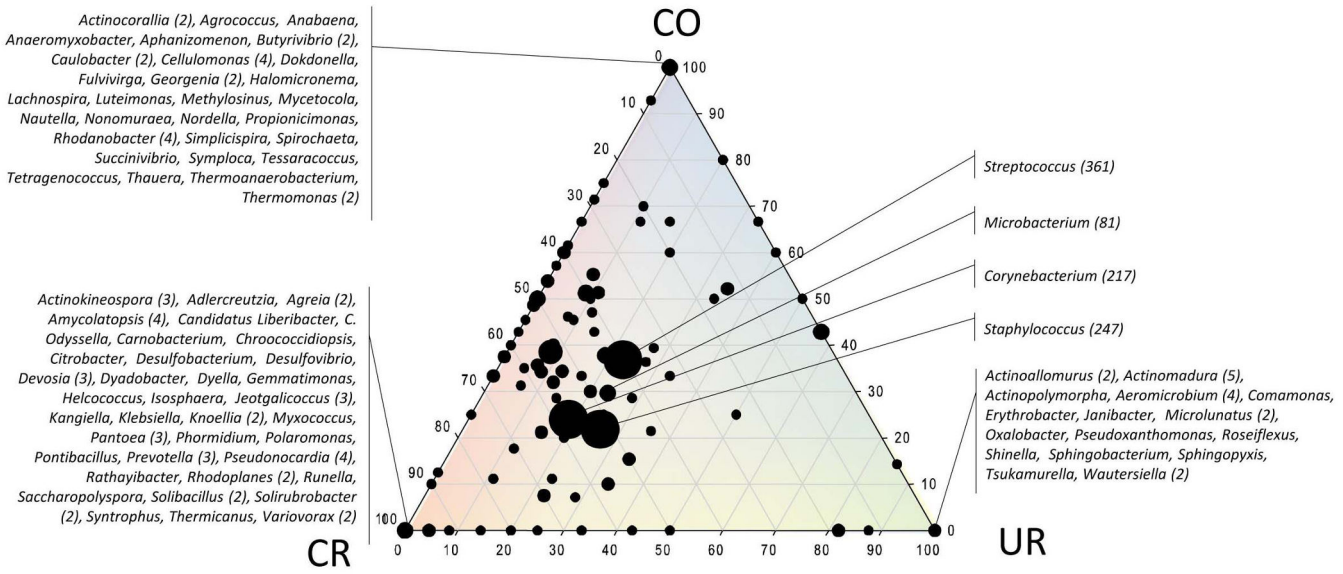

Figure 6 | Ternary plots of detected rOTUs (PhyloChip, non-PMA treated sample) with respect to the sample origin (the two cleanrooms CR5 and CR8 were summarized: CR). Axes reflect the percentage of OTUs detected in each location; OTUs that could not be attributed to a order, family or genus were not considered for calculation. Size of dots reflects no. of detected OTUs summarized in one dot (no. given in brackets if different from 1).

and UR areas (see Fig. 4 and Table S5). Overall, the largest portion of *Firmicutes* sequences was obtained from cleanroom samples.

In order to find microorganisms that increased or declined in cleanroom samples, read abundances were normalized to 5000 across each sample and then aggregated at genus level. Genera exhibiting at least 10 reads in one sample and showing a decrease or increase of at least 25% in cleanroom samples over non-cleanroom samples (tested individually) were filtered from the entire dataset and are depicted in Fig. 5.

Altogether 44 microbial genera were found to vary greatly between the two room categories, 17 of them decreased in non-cleanroom samples. These 17 included many Gram (−) bacteria like *Proteobacteria*-related taxa but also *Actinobacteria*. Most of the microbial taxa enriched in cleanroom samples were designated Gram (+), like *Firmicutes* (clostridia, *Paenibacillus*) and again *Actinobacteria*.

PhyloChip G3™ DNA microarray revealed variations in microbial richness and a great reduction of *Staphylococcus* and other genera in cleanroom areas when considering signatures from intact and non-intact cells. Presence/absence calling of reference-based operational taxonomic units (rOTUs) produced values ranging from 2 to 1007 different microbial taxa with 2059 different rOTUs in total. All areas revealed the signatures of *Streptococcus*, *Microbacterium*, *Corynebacterium* and *Staphylococcus* with up to 361 detected rOTUs belonging to *Streptococcus* (non-PMA treated samples; Fig. 6). Considering the microbial diversity that was unique for each facility area, PhyloChip analyses revealed different compositions compared to pyrotagsequencing data with the exception of *Simplicispira* and *Helcococcus* sequences, which were found by both methods to be solely present in CO and CR, respectively. A complete list of all detected phylotypes (PhyloChip) is given in Table S6.

In order to detect the intact (and thus probably living portion of microbial contaminants), PhyloChip was combined with PMA-treatment prior to DNA extraction of each sample[29]. Non-PMA samples generally exhibited more than 500 different rOTUs (511 to 1007), whereas samples treated with PMA had a much lower microbial richness ranging from 2 to 190 different rOTUs. A statistical comparison (paired student's t-test) of PMA treated to non-PMA samples resulted in a p-value of <0.005 indicating a highly significant reduction of the microbial richness in PMA treated samples. On abundance level, OTUs were analyzed with regard to increase after PMA treatment. Here, 14 different rOTUs produced a significant p-value (<0.05, paired student's t-test), which all belonged to the phylum *Proteobacteria* in the class *Betaproteobacteria/Gammaproteobacteria*. The 14 rOTUs were classified as *Bradyrhizobiaceae*, *Phyllobacteriaceae*, *Erythrobacteraceae*, *Sphingomonadaceae*, and *Pseudomonadaceae*. Consequently, the abundance of these rOTUs was underestimated when non-PMA sample data were analyzed. Focusing on microorganisms that get selectively reduced due to cleanroom conditions, rOTU abundances were first rank-normalized across each array and then aggregated at genus level. Genera that decreased or increased at least 25% in their relative rank in both cleanroom samples compared to both non-cleanroom samples were filtered from the entire genus dataset. These genera are displayed in Fig. 7 and belonged to various phyla. Since PMA and non-PMA samples were treated separately, some rOTUs showed an increase in PMA samples but a decrease in non-PMA samples. This effect can be attributed to corresponding amount of DNA signatures from non-intact cells in the samples, which could have a masking effect. Fig. 7 depicts 48 different genera, which showed some congruence with the pyrotagsequencing predicted changes (e.g. *Paenibacillus*). However, when considering the PMA-treated samples, information regarding the reduction of microbial signatures due to potential cleaning efforts can be gained. For instance, when considering only the intact fraction of cells, staphylocci were enriched in the less controlled environments of the changing room and the checkout room. In contrast, the non-PMA samples exhibited similar aggregated ranks of *Staphylococcus* signatures in cleanroom and changing room samples, while only the checkout room exhibited less prominent signatures. Consequently, *Staphyloccus* appeared to get reduced due to the controlled environment of cleanrooms.

The changing room revealed the lowest diversity but the highest abundance of microbial signatures. For a comparative analysis of 16S rRNA gene cloning, pyrotagsequencing and PhyloChip G3 technology representative sequences of OTUs were classified with the same taxonomic tool against the same database (see Methods for details). Measures of microbial diversity of pyrotagsequencing and PhyloChip G3 showed that the changing room harbored the lowest

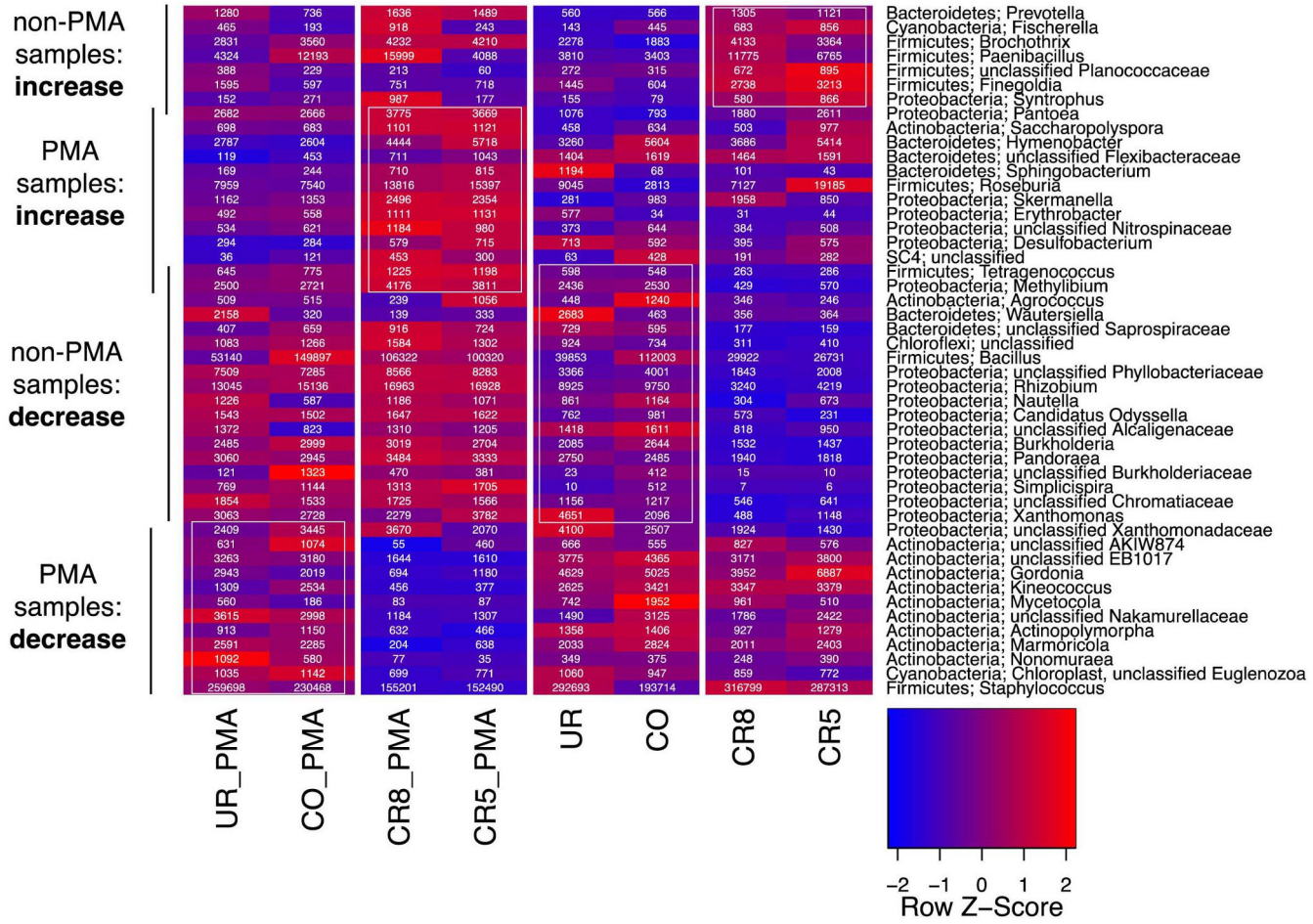

Figure 7 | Heatmap based on summarized OTU trajectories (genus level) derived from PhyloChip G3™ data (after rank normalization of rOTUs). Displayed are genera that showed at least 25% increase or decrease in both cleanroom samples compared to non-cleanroom samples. White boxes indicate taxa that showed a 25% increase over other corresponding samples. Numbers in the cells give the summarized rank for each genus. For the color gradient, rank scores were normalized for each genus and are presented as Z-scores. Non-PMA and PMA treated samples are displayed individually.

diversity in the cleanroom facility (Shannon-Wiener indices are provided in Table 1).

PMA pretreatment (detection of intact cells) was performed for experiments with PhyloChip G3 and 16S rRNA gene cloning. PMA treated samples, which were analyzed by PhyloChip G3, revealed a significant decrease in their diversity indices compared to the total microbial fraction (p-value 0.026, paired student's t-test). Concerning microbial richness measure, no correlation of number of OTUs in non-PMA treated samples was found when comparing the different methodologies (p-value > 0.05). However, when the OTUs were grouped at genus level, OTUs derived from PhyloChip G3 experiments (rOTUs) and pOTUs (OTUs obtained from pyrotagsequencing) showed a significant correlation of the microbial richness measure ($p = 0.003$, Pearson's $r = 0.997$, Fig. 8). A paired student's t-test testing for differences between genus richness of PMA and non-PMA samples produced a significant result for cloning ($p = 0.011$) and highly significant result for PhyloChip G3 data ($p = 0.001$). Thus, PMA-treated samples clearly show different richness than non-PMA samples. With regard to the agreement of PhyloChip G3™ and pyrosequencing, 62% of all genera detected by PhyloChip G3 technology were also detected via 454 pyrosequencing as depicted in Fig. 8. 16S rRNA gene cloning revealed seven genera, which were not detected by PhyloChip G3 or 454 pyrosequencing. Fig. 9 displays the microbial richness of genera detected in each sample grouped at phylum level (class level for *Proteobacteria*). The changing room (UR) generally showed the lowest amount of different genera detected by all three methods employed. However, as found with cultivation-dependent and -independent methods, the changing room (UR) revealed the highest contamination level with respect to colony forming units and detectable 16S rRNA genes after PMA treatment (Table 1).

All methods revealed different microbiomes present in controlled and uncontrolled areas. Adonis testing (Refs. 27, 28) based on abundance metrics produced a significant p-value for PMA versus non-PMA samples (0.034 for cOTUs (cloning), 0.036 for rOTUs (PhyloChip G3); experiment was not performed for pyrosequencing) indicating that PMA treated samples harbored a different microbiome structure than non-PMA samples. Ordination analyses based on rank-normalized abundance scores of cOTUs and rOTUs (Fig. 10) showed a separation of PMA-treated samples. This is in accordance with the above mentioned significant p-value in the Adonis test. Moreover, ordination analysis showed for all three methods employed (16S rRNA gene cloning, pyrosequencing and Phylochip) that samples taken from cleanrooms (CR) group together apart from other samples (check out room (CO), changing room (UR)) considering PMA treated and non-PMA samples separately. Similar observations were made for HC-AN analysis with the exception of clone library data, which were, however, only based on few counts in the PMA-treated samples.

The archaeal microbiome was predominated by *Thaumarchaeota* representatives. Archaeal 16S rRNA gene signatures were detected for each locations, whereas the CR5 facility revealed slightly higher

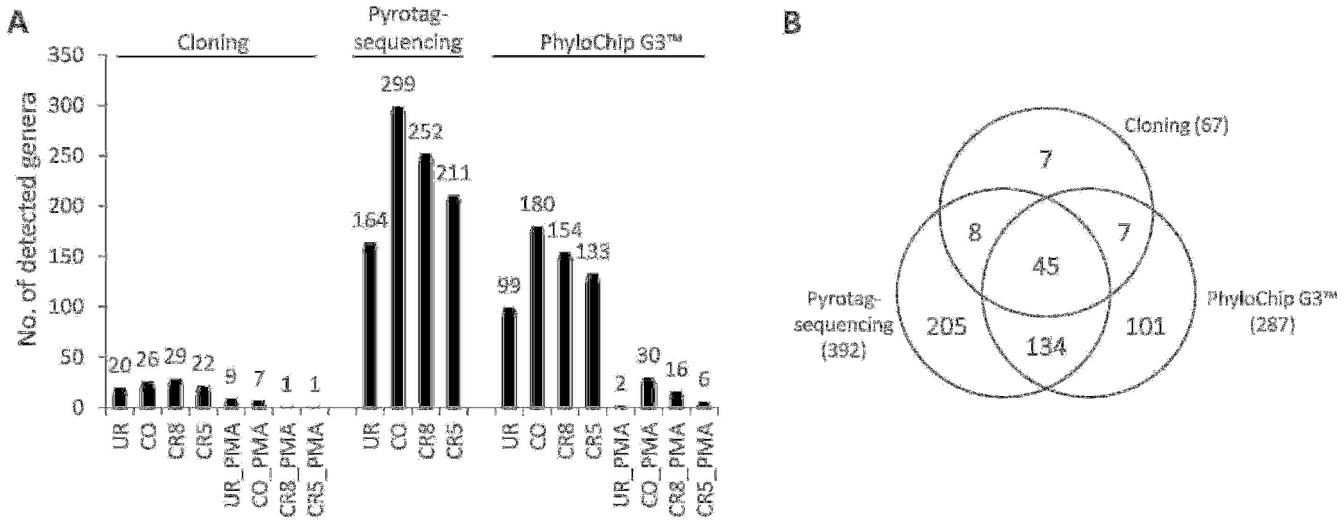

Figure 8 | Comparison of molecular methods. All sequences were classified using the same method (Bayesian method in mothur, GreenGenes taxonomy) as indicated in Materials and Methods. (A): Richness comparison of genera detected in each sample via cloning, pyrotagsequencing, and PhyloChip G3. While no significant correlation was detected between cloning and pyrotagsequencing/PhyloChip richness, pyrosequencing and PhyloChip derived genus richness correlated highly significantly between samples (p-value = 0.003, Pearson's R = 0.997). No significant correlation was found for OTU based richness (data not shown). (B): Venn-Diagramm displaying the shared genera between the three techniques used in this study.

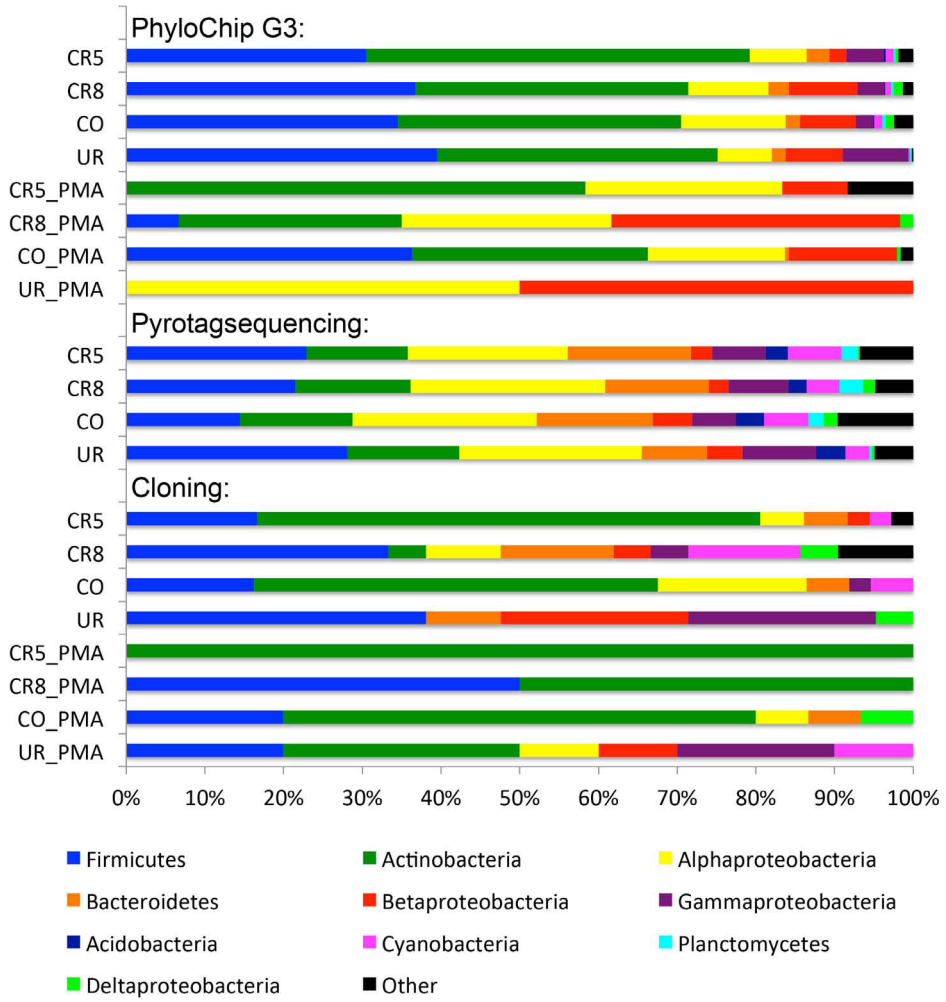

Figure 9 | Barchart displaying the percent richness of c/p/rOTUs classified at higher taxa (phylum level, for Proteobacteria class level, incidence values of OTUs), for each sample and analysis method. All sequences were classified using the same method (Bayesian method in mothur, GreenGenes taxonomy) as indicated in the Methods section. The top ten most prominent higher taxa are shown ascending while the remaining taxa are grouped into category "other".

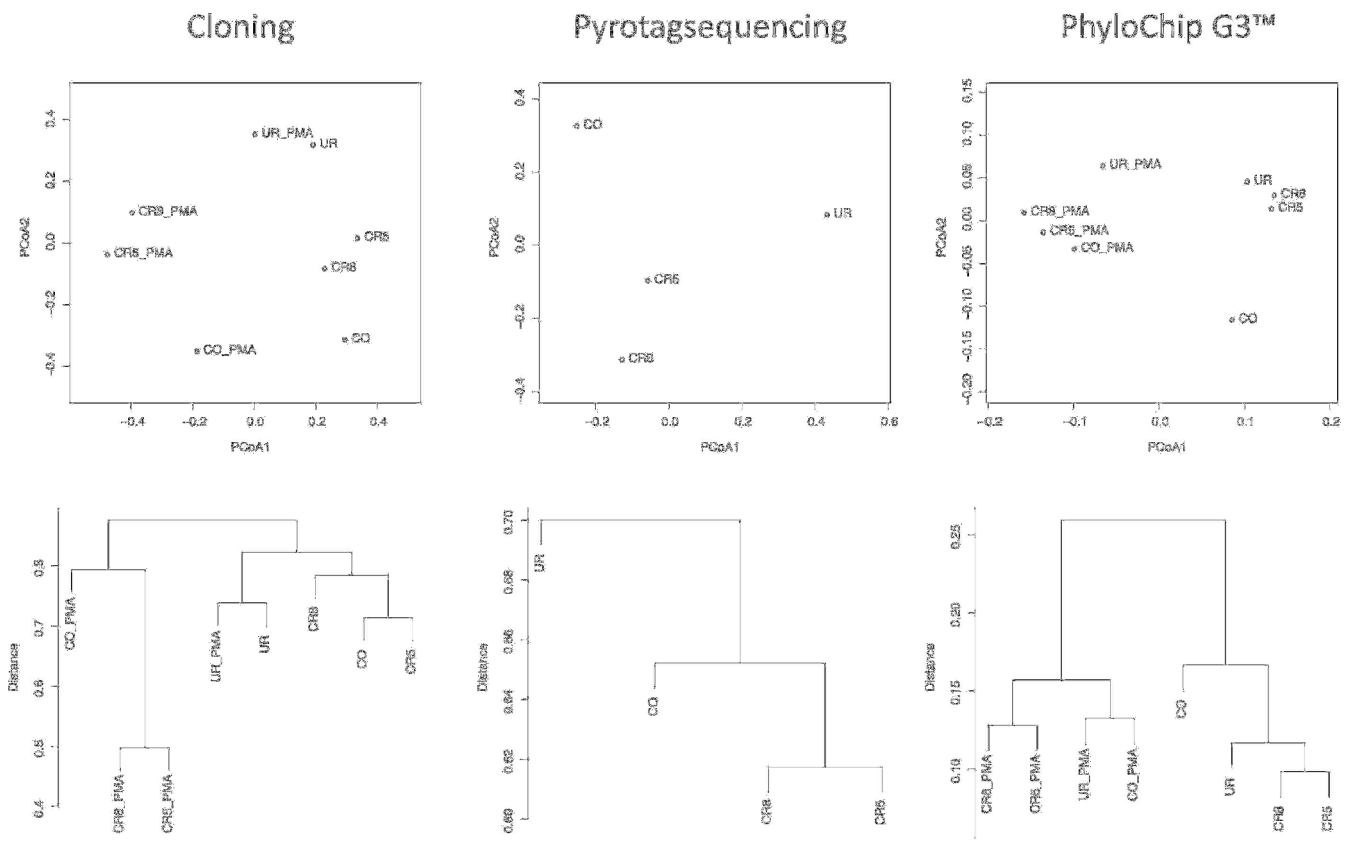

Figure 10 | Ordination analysis and hierarchical clustering (average neighbour) of cloning, pyrosequencing and PhyloChip G3™ derived bacterial microbiomes. Analyses are based on Bray-Curtis indices of rank-normalized abundance scores of OTUs. Explained variances of PCoA axes were 29% (PCoA1) and 19% (PCoA2) for cloning, 40% (PCoA1) and 33% (PCoA1) for pyrosequencing, and 66% (PCoA1) and 13% (PCoA2) for PhyloChip data.

qPCR signals than CR8 (1.7×10^5 and 0.9×10^5, respectively[25]). The archaeal diversity was investigated by pyrotagsequencing of 16S rRNA gene amplicons (Table S7 and included figure). OTU grouping revealed five (CR5) to 19 OTUs (CO), which were assigned to two archaeal phyla (*Thaumarchaeota* and *Euryarchaeota*, Table S7). The dominant lineage (*Candidatus* Nitrososphaera) accounted to 55–92% of all reads of each location. Signatures of halophilic archaea (*Halobacteriaceae*) were found in all sampled rooms, whereas *Halococcus* signatures appeared highly abundant in CR8 (43%). Signatures of *Methanocella* were detected in the check-out facility (CO). Cloning of 16S rRNA genes revealed the presence of signatures from unclassified (Eury)archaeota in CR8 as well as from Candidatus *Nitrososphaera* (both cleanrooms). Halophilic archaea have not been detected by 16S rRNA cloning[25].

Network analyses allowed tracking of the microbial routes and identified the changing room as most critical contamination source for the cleanrooms. All sampled rooms shared certain OTUs as presented in the network analyses (see supplementary Fig. S1 for pyrotagsequencing and supplementary Fig. S2 for PhyloChip analysis). Network tables were generated in QIIME (see Material and Methods and supplementary real node and edge tables S9.1, S9.2, S10.1 and S10.2) and visualized in Cytoscape. Lower amounts of pOTUs were shared outside the cleanroom (CO and UR, 18 pOTUs), than inside cleanrooms CR8 and CR5 (39 pOTUs). pOTUs detected in UR were spread to the highest relative proportion (68%) throughout the cleanroom facility. Although high in relative abundance and taxonomic resolution only a few pOTUs were common in all four sample locations (68 pOTUs), many were grouped at two (204 pOTUs) or three locations (115 pOTUs). The network revealed a similar portion of exclusive pOTUs in both cleanrooms (208 pOTUs in CR8 and 180 pOTUs in CR5), in contrast to CO and UR, where CO showed the highest (411 pOTUs) and UR the lowest number (76 pOTUs) of exclusive pOTUs. Similar patterns could be observed for rOTUs derived from PhyloChip data with the following exceptions: Most rOTUs were common in two sample locations (654 rOTUs). Portions of exclusive rOTUs were highest in CR8 (393 rOTUs) followed by CO (356 rOTUs) and lowest in CR5 (185 rOTUs) and UR (174 rOTUs). Beside UR, rOTUs were spread to the highest relative proportion in CR5 as well (~66% both rooms). Additional patterns were detected by the use of PMA treatment of samples. Hence, rOTUs from UR spread high, but only the smallest fraction (compared to all other samples) were derived from uncompromised cells (14% relative proportion). In contrast, almost all rOTUs from CR8 were represented by intact cells (59% CR8_PMA compared to 61% CR8).

Discussion

HEPA air filtration, control of humidity and temperature, partial overpressure (ISO 5), frequent cleaning, limited number of persons working at the same time in a cleanroom and strict changing protocols – all these cleanroom maintenance procedures have strong impact on the abundance, viability and diversity of microorganisms therein. Such countermeasures, performed in order to decrease particulate contamination, result in the development of clearly distinct microbial communities in controlled and uncontrolled facility areas.

Firstly, the abundance of molecular microbial signatures and colony forming units was tremendously reduced within the cleanrooms

compared to changing and office area. This has been proven true via four different methods. The changing room revealed the highest CFU numbers in all cultivation assays (except heat-shock resistant bioburden) and the highest number of 16S rRNA gene signatures per m^2 (PMA-qPCR), whereas lowest numbers were detected in CR5 in these experiments (except cultivation of alkaliphiles, which also revealed 0 in CR8). The microbial abundance with respect to CFU thus decreased from UR to CR5 by a factor of 43 (oligotrophs), 431 (alkaliphiles), 444 (anaerobes), 10 (heat-shock resistant bioburden), and the 16S rRNA gene numbers by a factor of 6 (qPCR) and 40 (PMA-qPCR; Table 1). Secondly, the portion of intact cells decreased immensely: Only 10% (CR8) and 1% (CR5) of the qPCR signals obtained from the cleanroom samples were judged to be derived from intact, and thus possibly living cells. These values are in in the range of previously reported numbers for cleanroom facilities[29]. However, the ratio of these probably living cells was tremendously higher for the changing room (UR; 45%), which is in congruence with the cultivation-based experiments, revealing a decrease of the cultivable microbial portion towards cleanrooms by at least 10 fold. Thirdly, the cleanroom areas are most likely highly influenced by the human microbiome. Although each investigated room harbored its indigenous microbiome, a low, but general overlap of microbial diversity was found. In particular *Staphylococcus, Micrococcus, Corynebacterium, Propionibacterium, Clostridium*, and *Streptococcus* were detected by different methods in all facility areas, implying the major source of bacteria in these facilities: the human body. Fourthly, the cleanroom maintenance procedures clearly impacted the microbial diversity. Cultivation experiments revealed several microbial genera, which were exclusively found in the cleanrooms, including *Staphylococcus* (*S. lugdunensis, S. pettenkoferi*), *Erwinia* and *Cellulomonas*. Noteworthy, *S. lugdunensis*, a typical human skin commensal[25,30], did not appear in any other area except CR5. This finding indicates the presence of a potential "hot spot" for these microorganisms and an increased contamination risk via human activity in this area. Although staphylococci are clearly human-associated and thus might not embody a risk for planetary protection considerations, their presence could have severe influence on planetary protection bioburden measurements: Cleanroom *Staphylococcus* species were shown to be able to survive heat-shock procedures which are the basis for contamination level estimations[31]. However, when comparing microarray data from intact versus non-intact cells, a strong decrease of *Staphylococcus* signatures was found for cleanroom samples, although their diversity was even higher in these areas.

The changing room represents the area of highest human activity and agitation, compared to office area and cleanrooms. In the changing area, particles and microorganisms, attached to human skin or cloths (also brought from the outer environment), are spread all over the place: into the air and onto the surfaces. Consequently, the highest abundance of 16S rRNA gene signatures from intact cells was detected in this area (1.3×10^7 16S rRNA gene copy numbers per m^2). Noteworthy, this location also revealed the lowest microbial diversity when PhyloChip and pyrotagsequencing were applied. This finding, however, was not supported by cultivation-based experiments, pointing at a methodical problem of molecular techniques with microbial communities predominated by one or several species, which may arise from the various normalization procedures applied for these technologies. The central and important role of the changing area has been confirmed by network analyses, which revealed this location being the major source for microbial contamination possibly leaking into the cleanrooms. The changing procedure follows strict rules, thus being a completely defined and effective process to reduce the microbial (and particulate) contamination of cleanrooms. However, the microbial transport via this route, at least in our setting, could not completely be avoided. Interestingly a high portion of microbes transferred from the changing area into the cleanroom environment might be hampered to proliferate under these new extreme conditions, as revealed by network analyses of PhyloChip data. However after this selection process almost all microbes detected in the cleanroom environment (CR8) comprise intact cells (or spores), which now have a high potential to colonize new environments and products (e.g. spacecraft). As known from other studies, slight modifications in room architectures can have enormous impact on the indoor's microbiome und could help to further reduce the microbial contamination[8]. Thus, a two-step changing-room system, as it is generally established for cleanrooms of higher cleanliness levels, is certainly more effective in microbial contamination reduction. Studies of those systems, however, have not been conducted thus far.

To understand the introduction of contaminants and to estimate the risk of detected microorganisms for planetary protection or – under certain circumstances - even staff health, the natural origin as also the potential pathogenic character of the contaminants is of general interest. The genera detected via pyrotagsequencing in samples from uncontrolled environments were mostly assigned to natural environments. Particularly, soil-related genera were detected in the changing area. Noteworthy, 8% of the signatures detected in CO could be attributed to a food source. The cleanest area revealed sequences mostly from unknown sources (55%), and the lowest level of soil associated microorganisms (11%). Most bacteria with pathogenic potential were detected in UR (31%), followed by genera from the cleanroom environment (18%). The checkout room (CO) microbial community revealed the lowest pathogenic potential (13%). Relative proportions of potential beneficial microbes were higher in UR and the cleanroom CR8 (both 17%) than the checkout room CO (9%) and CR5 (7%). Interestingly, some beneficials belonging to the order of *Lactobacillales* like *Lactobacillus* and *Lactococcus* increased towards the cleanroom, and could also be associated with the human body[13,31].

Members of *Bacillus*, *Staphylococcus* and *Deinococcus* (identified in the cleanroom area) are well-known for their capability to resist environmental stresses[32,33]. With regard to clinical environments, the reduced diversity within such areas could lead to a proliferation of bacterial species with pathogen potential and might increase the risk to acquire disease or allergic reactions[34]. This knowledge offers the possibility to use ecological knowledge to shape our buildings in a way that will select for an indoor microbiome that promotes our health and well-being. Biocontrol using beneficials like lactobacilli or the implementation of a highly diverse synthetic beneficial community would be an option, which should be evaluated for indoor areas besides cleanroomrooms[35,36]. Each human activity is correlated with microbial diversity; therefore sterility in cleanrooms is impossible. This requires new ways of thinking and is also important for cleanroom facilities for pharmaceutical and medical products but also for hospitals, especially intensive care units.

In our comprehensive study, using cultivation-dependent and cultivation-independent methods, we obtained further insights into the microbiology of cleanrooms. We were able to show a strong effect of cleanroom maintenance procedures on diversity, abundance and physiological status of microbial contaminants. All rooms belonging to the cleanroom facility, an office, a changing room and two cleanrooms of different ISO certification (ISO 5 and 8), harbored different microbial communities, including non-intact and intact (thus possibly living) cells. Additionally, we revealed also potential contamination sources and routes within the facility and thus identified the changing room as the area harboring the major risk for cleanroom contamination. Currently used countermeasures to avoid a severe contamination with outside- microorganisms seem to work properly, but potential risks could highly be reduced by a different architecture of the changing area.

Methods

Sampling sites and setting. Sampling took place in September 2011 in Friedrichshafen, Germany. Samples were taken at various places within a cleanroom facility (integration center) maintained by the Airbus Defence and Space Division

(the former European Aeronautic Defence and Space Company, EADS). In this facility, different types of indoor environments were located in close vicinity as depicted in Fig. 1. Check-out room (office and control room, CO), changing room (change room with lockers and bench, directly attached to the entrance (air lock) of the cleanrooms, UR), ISO 8 cleanroom (H-6048, CR8) and ISO 5 cleanroom (to be entered through the ISO 8 cleanroom, CR5). Both cleanrooms were maintained according to their classification (ISO 14644; HEPA air filtration, control of humidity and temperature) and were fully operating. Particulate counts in cleanroom ISO 8 determined within three days before sampling did not exceed 10.000 particles (0.5 μm) and 100 particles (5.0 μm) per ft^3 (~0.028 m^3), respectively, and therefore exhibited contamination levels well within specifications. Cleanroom ISO 5 was maintained with overpressure. These indoor environments reflect different levels of human activity, presence of particles (CO, UR: uncontrolled; CR8: 3.5×10^6 and CR5: 3.5×10^3 particles $\geq$ 0.5 μm), clothing (CO: streetwear; UR: changing area; CR8 cleanroom garment; CR5 complete covered cleanroom garment), entrance restrictions (CO to CR5 increasing restrictions), cleaning regimes (CO and UR household cleaning agents; CR8 and CR5 alkaline cleaning agents or alcohols) and environmental condition controls (CO and UR uncontrolled conditions; CR8: 0.5 air change per min, filter coverage 4–5%, filter efficiency 99.97%, vinyl composition tile on floors; CR5: 5–8 air change per min, filter coverage 60–70%, filter efficiency 99.997%, vinyl or epoxy on floors). As given above, sample abbreviations were as follows: CO (check-out room), UR (changing room), CR8 (ISO 8 cleanroom), CR5 (ISO 5 cleanroom).

Sampling and sample processing. All areas (CO, UR, CR8, CR5) were sampled individually and in parallel. Samples were collected from floor (areas of 1 m^2 maximum (1 sample) and 0.66 m^2 (all other samples)) by using BiSKits (biological sampling kits, Quicksilver Analytics, Abingdon, MD, USA) for molecular-based analysis and wipes (TX3211 Sterile Wipe LP, polyester; Texwipe, Kernersville, NC, USA; 15 × 15 cm; wipes were premoistened with 4 ml of water before autoclaving) for cultivation-based assays. Overall, 74 samples were taken (see supplementary Fig. S3). BiSKit samples (four from each room) for molecular analyses were pooled according to the area sampled and immediately frozen on dry ice. Wipe samples (four per location for bioburden analysis, eight per room for alternative cultivation strategies) were stored on ice packs (4–8°C) and microbes were extracted immediately after return to the laboratory for inoculation of cultivation media (within 24 h after sampling). In sum, 10 field blanks were taken as process negative controls.

Cultivation. Wipes were extracted in 40 ml PBS buffer (for sampling, extraction and cultivation procedures of anaerobes: please refer to Ref. 34. For the cultivation of oligotrophic microorganisms, 5 × 1 ml of the sample was plated on RAVAN agar (including 50 μg/ml nystatin; Ref. 34). Alkaliphilic or alkalitolerant microbes were grown on R2A medium, pH 10 as given earlier (Ref. 24). Facultatively or strictly anaerobic bacteria were cultivated on anoxic TSA plates[36]; 4 × 1 ml was plated and plates were incubated under nitrogen gas phase. Incubation was performed at 32°C for 8 (alkaliphiles), 11 (anaerobes) and 12 days (oligotrophs), respectively. Additionally, the microbial bioburden was determined following the ESA standard ECSS-Q-ST-7055C (wipe assay for bioburden (heat-shock resistant microbes) and vegetative microorganisms). Sampling and wipe-extraction details were also described earlier (Ref. 29). In brief, wipe samples (in 40 ml water) were split into two portions, whereas one aliquot was subjected to heat-shock treatment (80°C, 15 min). Sample was pour-plated in R2A medium (4 × 4 ml). Samples for vegetative microorganisms (not subjected to heat-shock) were pour-plated similarly. Cultivation was performed at 32°C for 72 hours (final count).

Isolate processing and taxonomic classification. Isolates were purified by two subsequent streak-outs and sent to DSMZ (Leibniz institute DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). At DSMZ, strains were classified using MALDI-TOF MS (matrix assisted laser desorption/ionization time of flight mass spectrometry) or 16S rRNA gene sequencing. MALDI-TOF mass spectrometry was conducted using a Microflex L20 mass spectrometer (Bruker Daltonics) equipped with a N_2 laser. A mass range of 2000–20.000 m/Z was used for analysis. MALDI-TOF mass spectra were compared by using the BioTyper (Bruker Daltonics) software package for identification of the isolates. Currently the MALDI Biotyper reference library covers more than 2,300 microbial species. Strains which could not be identified by MALDI-TOF, were identified by 16S rRNA gene sequence analysis.

DNA extraction for molecular assays. Due to the low-biomass-nature of the samples and the recurrent observation of an inhomogeneous microbial distribution in cleanrooms (see also Ref. 31), the samples were pooled by facility room for molecular analyses (4 BiSKit samples per location) in order to allow a more accurate estimation of microbial diversity. Pooled BiSKits samples were thawed gently on ice over night and concentrated. 1/5 of each sample was treated with propidium monoazide (20 mM) as described elsewhere (Ref. 2) for masking free DNA. Covalent linkage was induced by light (3 min, 500 W). In general, all samples were subjected to bead-beating for DNA extraction (PowerBiofilm RNA Kit Bead Tubes, MO BIO, Carlsbad, CA, USA; 10 min vortex). Supernatant was harvested after centrifugation (5200× g, 4°C, 1 min) and bead-washing with 400 μl DNA-free water and subsequent additional centrifugation (100× g, 4°C, 1 min). DNA was extracted from PMA-treated and untreated samples using the XS-buffer method as described earlier[37]. The resulting pellet was solved in 15 μl DNA-free water.

Quantitative real-time PCR. QPCR was performed as described earlier[10]. One microliter of extracted DNA was used as template and amplification was performed with Bacteria- and Archaea-targeted primers using the SYBR Green system. As a reference, 16S rRNA gene amplicons of *Methanosarcina barkeri* (archaeon) and *Bacillus safensis* (bacterium) were used for generation of a standard curve. QPCR was performed in triplicates for each sample.

Cloning and sequencing of bacterial 16S rRNA gene amplicons. Cloning of archaeal and bacterial 16S rRNA genes was performed as described earlier (Ref. 31). For the analysis of bacterial 16S rRNA genes from PMA-untreated samples, each 96 clones were analyzed; additionally 48 and 72 clones were picked for samples from the cleanrooms (CR5 and CR8, respectively). 48 clones were analyzed from PMA-treated samples. Cloned 16S rRNA genes were RFLP analyzed (*Hin*fI, *Bsu*RI), representative inserts were fully sequenced and chimera-checked (Bellerophon[3]; Pintail[38]). The sequences were submitted to GenBank (accession nos: JQ855509-635) and grouped into operational taxonomic units (OTUs; later referred to as cOTUs). Coverage was calculated according to Good, 1953[39].

454 pyrotagsequencing analysis of bacterial and archaeal 16S rRNA genes. For bacterial diversity analyses, DNA templates from all four rooms were amplified using the bacteria-directed 16S rRNA gene primers 27f and 1492r (5 μM each[40]), followed by a second (nested-) PCR with tagged primer Unibac-II-515f_MID and untagged Primer Unibac-II-927r_454 (10 μM each[41]). Polymerase chain reactions were accomplished with Taq & Go ™ (MP Biomedicals) in 10 μl 1st PCR – and 30 μl 2nd PCR reaction mix as follows: 95°C 5 min, 30 cycles of 95°C 30 s, 57°C 30 s, 72°C 90 s and 72°C for 5 min after the last cycle; 32 cycles were applied for the 2nd PCR with the following parameters: 95°C 20 s, 66°C 15 s, 72°C 10 min. Archaeal PCR-products were obtained by nested PCR as described earlier[25]. The first PCR was performed using Archaea-directed primers 8aF and UA 1406R primers[42,43]. The second PCR included 5 μM primers (340F_454 and tagged 915R_MID[44,45]), 6 μl Taq-&Go™ [5×], 0.9 μl $MgCl_2$ [50 mM] and 3 μl PCR product of the first archaeal PCR in a final volume of 30 μl. An optimized temperature program for primers with a 454 tag included the following steps: initial denaturation 95°C 7 min, 28 cycles with a denaturation at 95°C for 30 s, annealing at 71°C for 30 s, elongation at 72°C for 30 s, repetitive cycles were concluded with a final elongation at 72°C for 5 min. After PCR, amplified products were pooled respectively and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, USA) according to manufacturer's instructions. Pyrotagsequencing of equimolar PCR products was executed by Eurofins MWG Operon (Ebersberg, Germany) on a Roche 454 GS-FLX+ Titanium™ sequencer. Resulting 454 reads (submitted to QIIME-DB, http://www.microbio.me/qiime/ as Study 2558) were analyzed using the QIIME[46] standard workflow as described in the 454 Overview Tutorial (http://qiime.org/tutorials/tutorial.html) and briefly summarized in the following: Denoising of pyrotagsequencing reads of the four samples (CO, UR, CR5 and CR8) resulted in 1003-5118 bacterial and 890-2725 archaeal sequences. OTUs (later referred to as pOTUs) were grouped at 97% similarity level using uclust[47] picking the most abundant OTU as representative. Sequences were aligned using PyNAST[48]. An OTU table was created after removing chimeric sequences (561) via ChimeraSlayer (reference: greengenes 12_10 alignment) and filtering the PyNAST alignment. All pOTUs detected in the extraction blank were removed as potential contaminants from the entire sample set, which resulted in 816-2982 sequences (267–855 pOTUs) for bacteria and 47-229 sequences (5–14 pOTUs) for archaea. pOTU networks were visualized using Cytoscape 2.8.3 layout edge-weighted spring embedded eweights[49].

PhyloChip G3™ DNA microarray analysis of bacterial 16S rRNA gene amplicons. The basic of PhyloChip G3™ data acquisition and analysis can be found in the supplementary information of Hazen et al., 2010[50]. In brief, bacterial amplicons were generated as described above for 16S rRNA gene cloning with primer pair 9 bf and 1406ur[40]. After quantification, amplicons were spiked with a certain amount of non-16S rRNA genes for standardization, fragmented and biotin labeled as described in the abovementioned reference. After hybridization and washing, images were scanned. Raw data processing followed the principle of stage 1 and 2 analysis described in Hazen et al., but with modified parameters. First, an updated Greengenes taxonomy was used for assigning rOTUs ("reference-based OTUs") to the probes[51]. Second, only those probes were included in the analysis that corresponded to the targeted 16S rRNA gene region of the amplicons generated with 9 bf and 1406ur primers. Third, at minimum seven probes were considered for an OTU and the positive fraction of scored versus counted probes was set to 0.92. The quartiles of the ranked *r* scores (response score to measure the potential that the probe pair is responding to a target and not the background) were set to $rQ_1 \geq 0.80$, $rQ_2 \geq 0.93$, and $rQ_3 \geq 0.89$ for stage 1 analysis. For stage 2, the *r*x values (cross-hybridization adjusted response score) was set to $rxQ_1 \geq 0.22$, $rxQ_2 \geq 0.40$, and $rxQ_3 \geq 0.42$. These adjusted parameters are considered sufficiently stringent for cleanroom diversity measures. Calculated hybridization values for each OTU were $\log_2$*1000 transformed. As different amounts of PCR product were loaded onto the chips (PCR reactions performed differently for each sample, particularly for those that were PMA treated) abundance values were rank normalized across each array and are referred to as hybridization scores/abundances.

Taxonomic classification of 16S rRNA genes. 16S rRNA gene amplicons were classified using the Bayesian method implemented in mothur (cutoff 80%, Refs. 52, 53) against an updated Greengenes taxonomy[51], which was manually curated and in

which OTUs were grouped at 98% similarity level. For taxonomic comparison of rOTUs (obtained from PhyloChip analysis) against amplicon generated OTUs (cOTUs, pOTUs), representative sequences of rOTUs were also classified with this method.

Microbial diversity measure. Shannon-Wiener indices were computed of all samples using the R programming environment[54]. Phlyochip G3 abundance data was multiplied with binary data, i.e. using abundance data of only those rOTUs that were called present in a sample. Abundance data of clone libraries, pryotagsequencing libraries and microarray data were individually rarefied to the lowest number of OTU abundances in the sample set and the Shannon-Wiener index was calculated for each sample. To avoid statistical errors originating from rarefication, the procedure was performed 1000 times and the average Shannon-Wiener index of each sample was calculated.

Cytoscape OTU networks. Node and edge tables (see supplementary Tables S9.1, S9.2, S10.1 and S10.2) for OTU networks were generated in QIIME and visualized in Cytoscape 2.8.3[49]. Shared OTUs were colored according to their presence in each sample (color mixtures were applied according to the color circle of Itten). OTUs as well as samples were displayed as nodes in a bipartite network. Both were connected via edges if their sequences were present in that sample. Edge weights (eweights) were calculated according to the sequence abundance in an OTU. For network clustering of OTUs and samples a stochastic spring-embedded algorithm was used with a spring constant and resting length. Nodes were organized as physical objects on which minimized force was applied to finalize the displayed networks.

Statistical analysis. Multivariate statistics were employed for microbial community analysis[54]. Bray-Curtis distance was calculated from the clone library, the pyrotagsequencing and PhyloChip G3 abundance data, which were all rank-normalized. Principal Coordinate Analysis (PCoA) and Hierarchical Clustering based on Average Neighbour (HC-AN) was performed to analyze the microbiome relatedness of the samples. Adonis testing was used to investigate if PMA treatment of samples had a significant effect on the microbial community structure observed. Paired student's t-test was performed to find significant difference between qPCR data of PMA and non-PMA samples.

Identification of enriched genera. HybScores of rank-normalized OTUs were aggregated at genus level for PhyloChip data. Considering pyrotagsequencing data, sum-normalized reads (5000 per sample) were summarized at genus level. In order to identify those genera that were enriched in cleanroom samples versus others, a 25% increase of aggregated scores was used as a threshold. In a similar manner, a 25% decrease of HybScores/sequencing reads was used as an indicator for genera that declined in cleanroom samples.

Controls and blanks for molecular analyses and cultivation. Control samples were included in each step of the extractions and analyses. Field blanks (procedure see: Ref. 31), extraction blanks (for BiSKit samples: unopened PBS included in the kit was used for extraction), water blanks and no-template controls (for PCR), as well as media blanks were processed. If not stated otherwise no signal or positive cultivation result was obtained thereof. For bacterial 16S rRNA analyses, detected OTUs (for cloning, pyrotagsequencing and PhyloChip) were removed from the entire analysis (Table S8). Bacterial copies detected in qPCR negative controls were subtracted from sample values.

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Author contributions

Conceived and designed the experiments: C.M.E., A.J.P. and G.B. Performed the experiments: A.K.A., A.J.P., A.M., L.T., Y.P., S.B. and R.P. Analyzed the data: A.J.P. and A.M. Contributed reagents/materials/analysis tools: G.A., K.V. and P.R. Wrote the paper: C.M.E., A.J.P., A.M. and G.B. All authors contributed to the manuscript revision.

Additional information

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Supplementary Figures to:

Quo vadis? Microbial profiling of vicinal office, changing and clean rooms reveal clearly different microbiomes and routes of contamination

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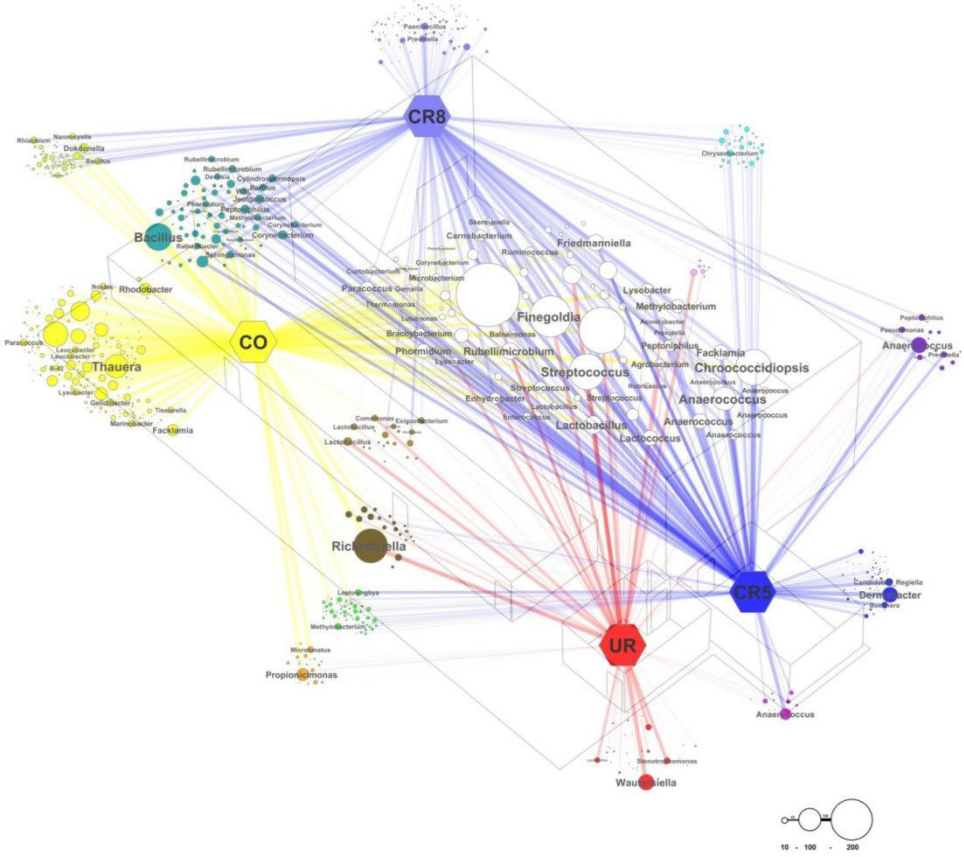
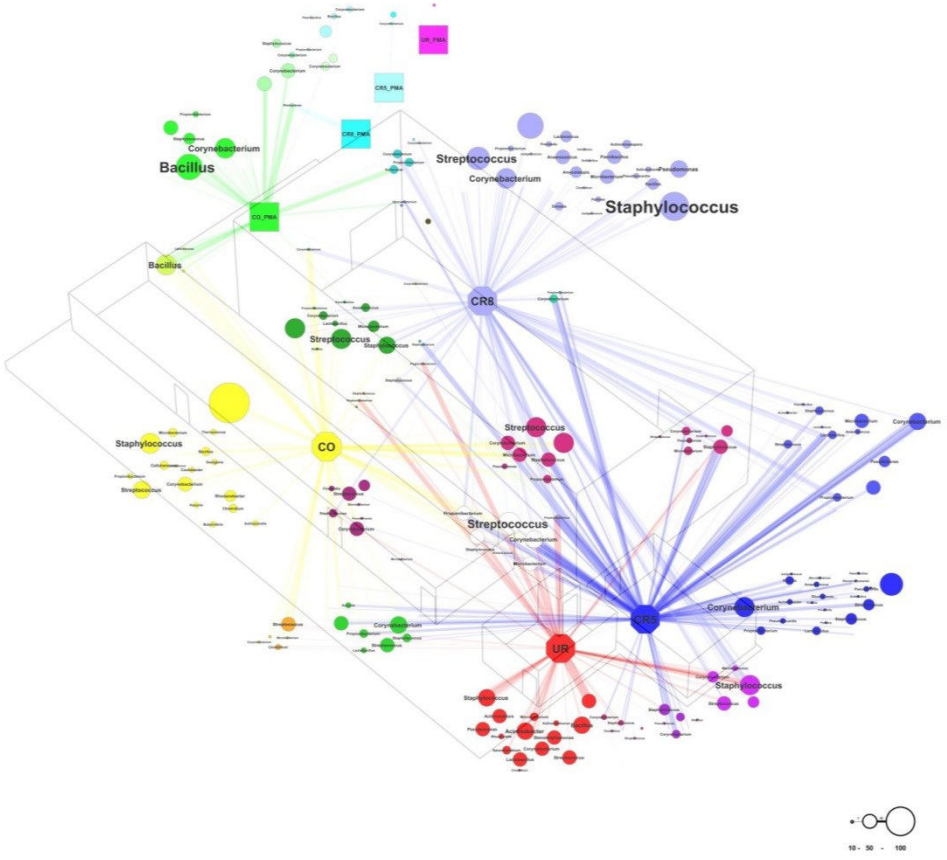
Supplementary Tables are accessible online

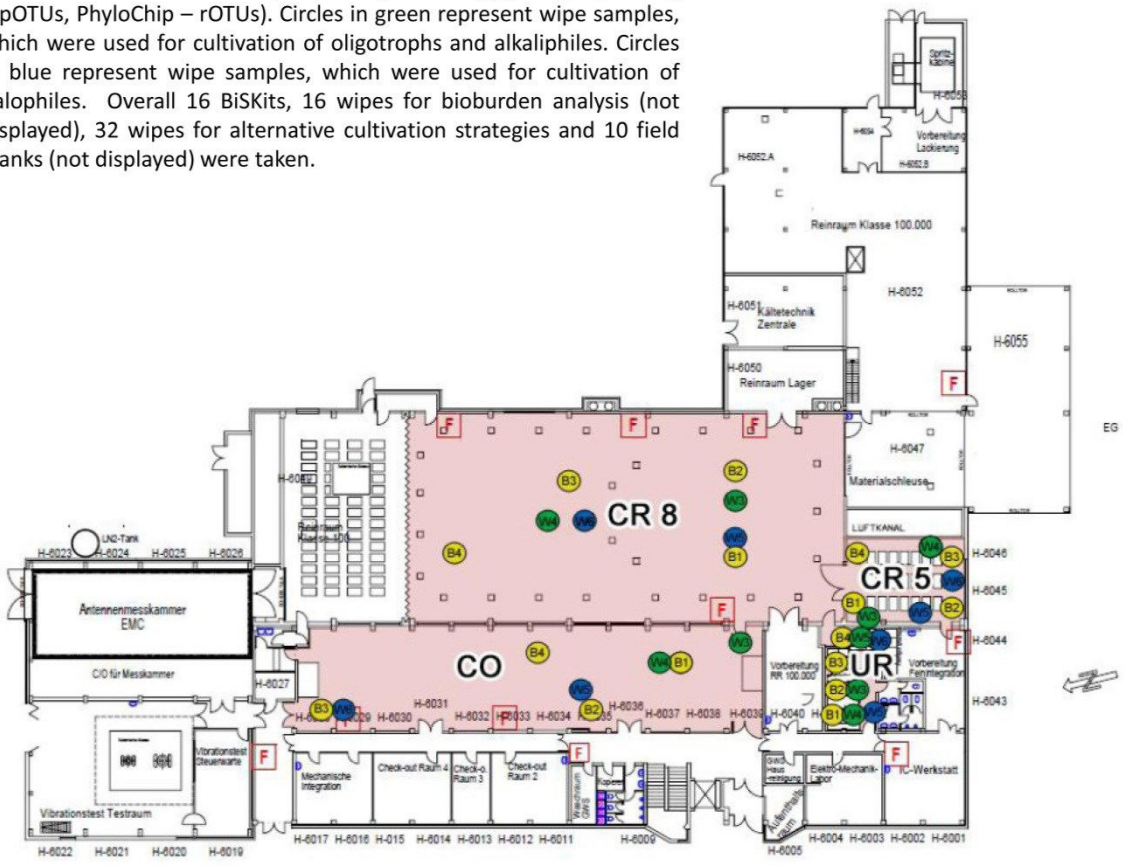
Supplementary Fig. S1:
Network analysis of pOTUs from pyrotagsequencing data visualized with the Cytoscape 2.8.3 layout edge-weighted spring embedded eweights. Samples from four different rooms are displayed as hexagonal nodes on an abstracted map of the clean room facility in colors yellow (CO – check out room), red (UR – changing room), light blue (CR8 – ISO8 clean room) and dark blue (CR5 – ISO5 clean room). pOTUs are displayed as nodes. Node color is defined by sample source and respective color mixtures (according to Itten's color circle) are used for shared pOTUs. pOTUs resolved till genus level are labelled. Node and font size represents abdundance of pOTUs (score is indicated in the legend below). Edge width and opacity correlates with respective eweights (edge weights represent sequence abundance in respective pOTUs). Network layout (pOTUs distance) was slightly modified to fit on the abstracted map.



Supplementary Fig. S2:
Network analysis of rOTUs from PhyloChip data (based on binary incidences table) using Cytoscape 2.8.3 layout edge-weighted spring embedded eweights. Samples from four different rooms are displayed as octagonal nodes on an abstracted map of the clean room facility in colors yellow (CO – check out room), red (UR – changing room), light blue (CR8 – ISO8 clean room) and dark blue (CR5 – ISO5 clean room) and their corresponding PMA treated samples are shown as squares in green (CO_PMA), magenta (UR_PMA) and cyan (CR8_PMA and CR5_PMA). rOTUs are displayed as nodes. Node color is defined by sample source and respective color mixtures (according to Itten's color circle) are used for shared rOTUs. rOTUs resolved till genus level are labelled. Node and font size represents abdundance of rOTUs (score is indicated in the legend below). Edge width and opacity correlates with respective eweights (edge weights represent sequence abundance in respective rOTUs). Network layout (rOTUs distance) was slightly modified to fit on the abstracted map.



Supplementary Fig. S3:
Layout of the integration center at Airbus Defence and Space GmbH in Friedrichshafen, Germany. Sampled rooms are highlighted in red and designated as follows: UR – changing room, CO – checkout room, CR8 – ISO 8 clean room, CR5 – ISO 5 clean room. Sample location were marked as follows: Circles in yellow represent BiSKit samples, which were used for molecular analysis (cloning – cOTUs, pyrotagsequencing – pOTUs, PhyloChip – rOTUs). Circles in green represent wipe samples, which were used for cultivation of oligotrophs and alkaliphiles. Circles in blue represent wipe samples, which were used for cultivation of halophiles. Overall 16 BiSKits, 16 wipes for bioburden analysis (not displayed), 32 wipes for alternative cultivation strategies and 10 field blanks (not displayed) were taken.



PLOS ONE

RESEARCH ARTICLE

Cleanroom Maintenance Significantly Reduces Abundance but Not Diversity of Indoor Microbiomes

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Abstract

Cleanrooms have been considered microbially-reduced environments and are used to protect human health and industrial product assembly. However, recent analyses have deciphered a rather broad diversity of microbes in cleanrooms, whose origin as well as physiological status has not been fully understood. Here, we examined the input of intact microbial cells from a surrounding built environment into a spacecraft assembly cleanroom by applying a molecular viability assay based on propidium monoazide (PMA). The controlled cleanroom (CCR) was characterized by ~$6.2*10^3$ 16S rRNA gene copies of intact bacterial cells per m^2 floor surface, which only represented 1% of the total community that could be captured via molecular assays without viability marker. This was in contrast to the uncontrolled adjoining facility (UAF) that had 12 times more living bacteria. Regarding diversity measures retrieved from 16S rRNA Illumina-tag analyzes, we observed, however, only a minor drop in the cleanroom facility allowing the conclusion that the number but not the diversity of microbes is strongly affected by cleaning procedures. Network analyses allowed tracking a substantial input of living microbes to the cleanroom and a potential enrichment of survival specialists like bacterial spore formers and archaeal halophiles and mesophiles. Moreover, the cleanroom harbored a unique community including 11 exclusive genera, e.g., *Haloferax* and *Sporosarcina*, which are herein suggested as indicators of cleanroom environments. In sum, our findings provide evidence that archaea are alive in cleanrooms and that cleaning efforts and cleanroom maintenance substantially decrease the number but not the diversity of indoor microbiomes.

Introduction

In recent years much attention has been given to the investigation of the microbiome of the built environment [1]. These are interesting habitats associated with human health [2–4] since humans spend most of their time indoors. In addition to its human residents [5,6] the microbiome of a built environment is determined by numerous environmental parameters: location, usage, architectural design, ventilation and occupancy, as important microbial dispersal vectors [1,7–11]. All studies show that humans are constantly exposed to microorganisms that might affect their microbiome and could potentially have a strong influence on health [12]. Although excessive removal and eradication of mainly beneficial microbes from the indoor environment could have adverse effects [13], some built environments like intensive care units, operating theaters, and particularly cleanrooms need to maintain indoor environments with very low microbial abundance to protect human health or to safeguard the quality of industrial product assembly. An enhanced understanding of the cleanroom microbiome can reveal the effect of microbial control, maintenance, and cleaning on microbial community structure in the built environment.

Cleanrooms represent highly specific environments because many of the environmental parameters are controlled e.g. the amount of particles (via cleaning, disinfection, surface sterilization), type and quality of gaseous substances (air quality is adjusted by filters and adsorption), temperature and humidity (using air conditioning systems), the light source, electrostatics and electromagnetics (can be controlled by deduction, ground connection, ionization and architectural arrangements) (for ISO certified cleanrooms see Online Browsing Platform on ISO standards; https://www.iso.org/obp/ui/#iso:std:iso:14644:-1:ed-1:v1:en). Cleanrooms used for spacecraft assembly have been monitored with special adapted sampling protocols for low-biomass environments in respect to microbial abundance and diversity for many years, as some space missions are subject to planetary protection policy [14–16]. However, complete sterility or absence of biological contamination in spacecraft assembly cleanrooms cannot be achieved during assembly, testing, and launching operations, since some resistant, well-adapted microorganisms are capable of surviving and withstanding harsh cleanroom conditions e.g. continuous HEPA air filtration and very low abundance of particles, water and nutrients [17]. Although humans were identified as the major vector for contamination in cleanrooms [18,19], trained engineers still have to interact closely with spacecraft components to guarantee assembly quality. Since Viking mission days, these cleanrooms have been studied and documented by traditional microbiological methods [20] and later cultivation independent molecular methods were employed as well to understand microbial community structure of the spacecraft and associated surfaces [21,22]. More recently, DNA microarray (PhyloChip) and high throughput sequencing technologies (pyrosequencing) were performed [23–26]; and showed that >90% of the sequences arose from dead and non-viable microbial cells by the application of propidium monoazide (PMA) treatment [25]. PMA is able to mask DNA from dead microbes with permeable cell structures and free extracellular DNA after light activation, for downstream PCR based analysis. Hence, the source of DNA can be determined for all and only intact cells [27].

Consequently, this study has been focused on the potential viable microbiome and its origin by a combination of the DNA masking agent PMA assay [27,28] with Illumina 16S rRNA gene amplicon deep sequencing and qPCR analysis. In addition, an adenosine tri-phosphate (ATP) assay that differentiates between dead organisms and measures only intact and metabolically active cells was used [29] and compared with the PMA-qPCR analysis. Since the changing room could be identified as the main source for microbial contaminants into the cleanroom environment in a prior study [26], we focused on the comparison of microbial abundance and diversity of intact cells between a controlled cleanroom (CCR) and its surrounding uncontrolled

adjoining facility (UAF). As a further step, deeper sequencing was applied to reveal the composition of underrepresented microbes and assess their viability status. This deep diversity analysis should add another puzzle to the picture of strict controlled built environments and estimate its risks to jeopardize future life detection missions.

Materials and Methods

Environmental conditions

The relatively small gowning area (45.9 m^2, Fig 1) was the main entrance for staff to have access into the particulate controlled cleanroom (CCR, spacecraft assembly facility–SAF at NASA Jet Propulsion Laboratory). Sticky mats in front of and behind doors were placed to remove dirt from the soles of shoes. The gowning area was equipped with lockers to keep the street clothes in before the donning of cleanroom garments. Before entering the air shower, personnel were required to clean their footwear by means of a shoe cleaner. Gowning with cleanroom certified textiles (coat, head cover, goggles, surgical mask, gloves, shoe-cover) was strictly followed and then staff members were allowed to pass through air shower systems before entering. Within the cleanroom, staff members were advised to perform their duties with slow body movements to avoid the spreading of human and floor associated particles. The cleanroom (ISO8, 100K, 921.1 m^2) itself had more than 20 times the floor surface compared to the gowning area. In both rooms, floor surfaces consisted of polymer plastic materials (vinyl composition tiles) and were illuminated by artificial light sources. Access to the cleanroom was strictly controlled, air was continuously exchanged through HEPA filter systems, temperature and humidity were constantly automatically adjusted, numbers of particles with a size $\geq$0.5 μm were counted, and floors were regularly cleaned up to several times per week. Sampling was performed prior to the regular cleaning schedule. Both rooms were cleaned on September 24th and on October 3rd 2012 using Kleenol 30 (Mission Kleensweep Products Inc., Los Angeles, USA). Kleenol 30 is a highly alkaline (11.5 to 12 pH) product, which consists of (v/v) 1% dodecyl benzene sulfonic acid, 1 to 4% silicic acid disodium salt, 12.5% ethanol, 1 to 5% nonylphenol ethoxylate, a non-ionic surfactant, dissolved in distilled water. During the period of sampling time the cleanroom did not harbor mission critical spacecraft hardware. The sampling team included four persons (three scientists and one person from the cleanroom managing staff). At the time of sampling, in addition to the sampling team, two assembly technicians were present in the CCR, whereas in the UAF only sampling team members were present. Particles smaller than 0.5 μm in size were far below the required specification for this CCR cleanroom (100K) during sampling operations (max. 570 particles per cubic foot; 20,129 particles per m^3).

Sampling procedure

While sampling, the ATP content was measured with a hand-held ATP device in real-time as described elsewhere [29] to identify hot-spots of microbial contamination and reveal the gross contamination of the surface to be sampled (Table A in S1 File). Subsequently, various cleanroom floor locations of the CCR and UAF were sampled. One square meter surface area of three adjacent places (A, B, C; Fig 1) of 10 different CCR locations was sampled. Each adjacent area of all 10 locations was pooled (3 replicates each containing 10 m^2) and analyzed for molecular analyses. Compared to UAF, ~10 times more surface area was collected from CCR, since it was reported that microbial abundance was ~10-fold less in a Class 100K (ISO 8) cleanroom compared to uncertified ordinary rooms [24]. Hence, when samples were taken from UAF only one square meter surface area of 4 different locations was sampled (Fig 1). Field blanks were collected without touching actual floor surfaces to reveal the microbial background contaminants from other sources than the floor surfaces (e.g. from the indoor air).

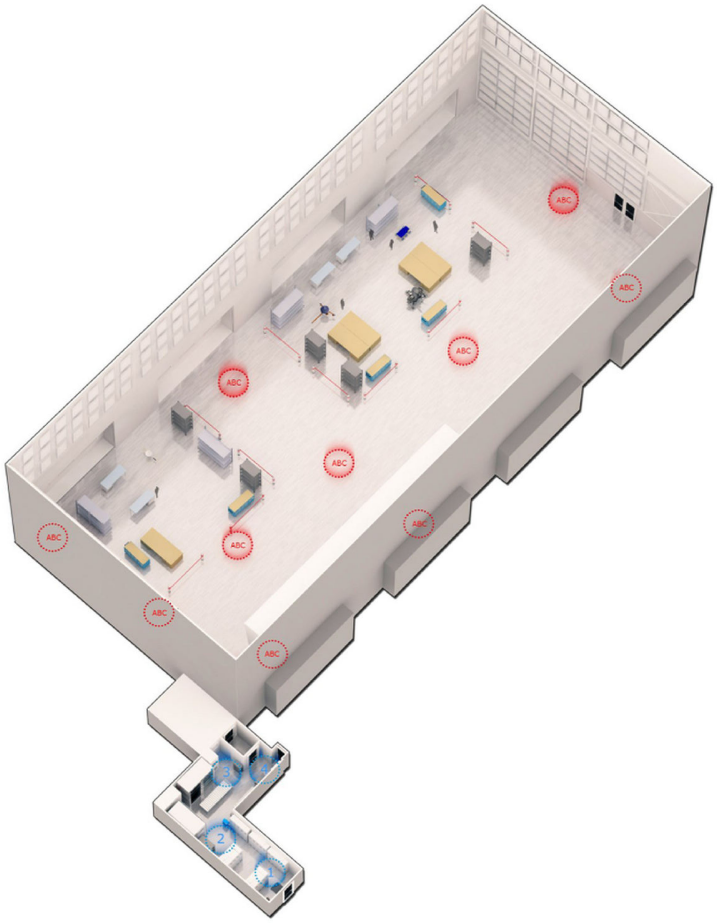

Fig 1. 3D-rendered-model. of sampled controlled cleanroom (CCR) and its uncontrolled adjoining facility (UAF). Triplicate of ten different locations in CCR are indicated by A, B and C (red circles). Samples from four individual locations in UAF are numbered 1 to 4 (blue circles).

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Surface sampling of the floor (1 m^2) was performed with adapted protocols for low-biomass environments using pre-moistened biological sampling kits (BiSKits; QuickSilver Analytics, Abingdon, MD) as described previously [30]. Prior to sampling, all the BiSKit devices were rinsed with 15 mL of DNA-free phosphate buffered saline (PBS), which served as controls throughout the experiments. The manufacturer-provided buffer was discarded and sterile PBS was used and then added to the macrofoam sponge component of the BiSKit. Once the PBS had adequately absorbed to the macrofoam sponge, the sampler was unscrewed from the module and was traversed about the surface area of interest (ca. 1 m^2), first in a horizontal fashion, then vertically, and finally in a diagonal sweeping pattern. Immediately following the collection of a sample from the surface, the macrofoam sponge sampler was forcefully screwed back into the BiSKit module so as to squeeze as much sample as possible from the sponge into the collection tube. The module and attached collection tube were then transported in a sealed bag to the laboratory, where they were further processed under a biohood. Upon return to the lab, the PBS was recovered from the BiSKit sponge by screwing the sponge casing against the BiSKit cover several times, allowing samples to be collected into the attached sample bottle.

Concentration of biomolecules

The pooled fractions of the samples were aseptically transferred to Amicon Ultra-15 centrifugal filter tubes (Ultracel-50 membrane, catalog no. UFC905096; Millipore, Jaffrey, NH), which were in turn placed within a Sorvall RC-5B refrigerated centrifuge (Thermo Scientific, Waltham, MA) and spun at 4,000 g for 10 min. Each filter unit has a molecular mass cutoff of 50 kDa, which facilitates the concentration of bacterial cells, spores, and exogenous nucleic acid fragments greater than 100 bp into a final volume of ca. 1.5 ml. The resulting volume was aseptically transferred to a sterile microcentrifuge tube. A comparable amount of sterile PBS was concentrated in a separate filter tube, serving as a negative control for each concentration/extraction.

Sample processing

All filtered samples were then divided into three separate aliquots: one of the aliquots (500 μl) was subjected to PMA pretreatment (viability assessment), the second (500 μl) was an untreated environmental sample (viable + nonviable cells; total DNA), and the third (500 μl) was used for ATP analysis (see below). One 500 μl aliquot of filter-concentrated sample suspension was treated with PMA (Biotium, Inc., Hayward, CA, USA) to a final concentration of 50 μM [27,31], followed by thorough mixing and incubation in the dark for 5 min at room temperature. The sample was exposed to PhAST blue-Photo activation system for tubes (GenIUL, S.L., Terrassa, Spain) for 15 min (in parallel with the non-PMA treated sample). Samples were then split in half and one half was subjected to bead beating with the Fastprep-24 bead-beating instrument (MP Biomedicals, Santa Ana, CA, USA) with parameters set at 5 m/s for 60 s. The second half of the unprocessed sample was then combined with the mechanically disrupted counterpart to allow microbial yields from hardy cells and spores with a limited loss of the overall microbial diversity. DNA was extracted via the Maxwell 16 automated system (Promega, Madison, WI, USA), in accordance with manufacturer's instructions, and resulting DNA suspensions (100 μl each) were stored at −20°C.

ATP-assay

A bioluminescence assay was performed to determine the total ATP and intracellular ATP from all samples using the CheckLite HS kit (Kikkoman), as described previously [29]. Briefly, to determine total ATP (dead and viable microbes), sample aliquots were combined with an equal volume of a cell lysing detergent (benzalkonium chloride) and incubated at room temperature for 1 min prior to the addition of a luciferin–luciferase reagent. The sample was mixed, and the resulting bioluminescence was measured with a luminometer (Kikkoman). For intracellular ATP measures of intact microbes a tenth volume of an ATP-eliminating reagent (apyrase, adenosine deaminase) was added to the sample and allowed to incubate for 30 min to remove any extracellular ATP. After extracellular ATP removal the assay for ATP was carried out, as described above, including sterile PBS as a negative control. As previously established, 1 Relative Light Unit (RLU), the unit of ATP measurement, was assumed to be approximately equal to 1 CFU [17].

Quantitative PCR

Quantitative PCR was accomplished as described [32]. One μl of extracted DNA (< 1 ng—> 10 ng) was used for a 20 μl reaction mix containing 10 μl SYBR Green Supermix (BIO-RAD, Hercules, CA, USA), 0.5 μl forward and reverse primers (1369F and 1492R; [33]) at a concentration of 18 μM and 8 μl of PCR grade water. DNA templates were amplified with 16S rRNA gene standards from *Bacillus subtilis* using a BIO-RAD C1000 qPCR thermal cycler in three

replications and the following program: 95°C for 3 min, 95°C for 15 sec., combined annealing and extension at 55°C for 35 s, steps repeated for 39 times and finished with a step at 95°C for 10 sec, and a final elongation from 65°C– 95°C by an increase of 0.5°C for 5 sec. respectively.

16S rRNA gene amplicon Illumina MiSeq deep sequencing: For 16S rRNA gene PCR, amplicons of samples and controls were generated with universal primers 515F and 806R [34–36] using 7–10 bp barcodes for identification in paired-end Illumina MiSeq runs [37,38]. One μl extracted DNA was used as a template in a 10 μl PCR reaction containing 5.5 μl PCR grade water, 2 μl 5xPhusion HF buffer, 1 μl 2 mM dNTPs, 0.2 μl of primers 515F and 806R (0.2 μM final conc.) respectively and 0.1 μl Phusion Polymerase. PCR settings were as follows: 98°C 30 s, 35 cycles of 98°C 10 sec, 50°C 30 sec, 72°C 10 sec and a final extension at 72°C for 10 min. Illumina barcodes were attached to PCR products in a subsequent 20 μl PCR reaction with 20 cycles of 98°C 10 sec, 60°C 30 sec, 72°C 11 sec. Barcoded products of four individual PCR reactions were pooled, cleaned with Wizard SV Gel and PCR Clean-Up System kit (Promega, Madison, WI, USA) and checked finally via gel electrophoresis. Resulting DNA concentrations were determined on the NanoDrop instrument (Thermo Scientific, Wilmington, DE, USA). Equimolar concentrations of amplicons were sent to LGC Genomics GmbH, Berlin, Germany for 2x 250 bp paired-end Illumina MiSeq deep sequencing using V2 chemistry. Sequences are deposited in the European Nucleotide Archive (www.ebi.ac.uk) under project PRJEB8763 (ERP009799).

Bioinformatics

Sequencing reads were demultiplexed with Illumina CASAVA analysis software. Adapters were clipped and reads with < 20 bp were removed. Corresponding forward and reverse reads were stitched into longer fragments using FLASH (overlap 10 bp, max. mismatch 0.25). Amplicons of samples and controls were further sorted by removing reads without barcodes, single reads (only one barcode) and barcode chimeras (different barcodes on 5 and 3 prime site). Resulting reads were quality filtered for deep diversity analysis with QIIME [39] at phred score q30 [40], 5'-3' orientated, labeled and additional quality filtered using default settings in QIIME. Reads in respective negative controls of each BiSKit sample were removed by a 100% aligned BLAST hit prior to OTU picking (see Table B in S1 File for details on all reads). OTUs were checked for chimeric sequences via ChimeraSlayer, clustered at 97% similarity level [41], taxonomy was assigned with the RDP classifier [42] and a phylogenetic tree was calculated [43]. The resulting rarefied OTU table served as a basis for following alpha and beta diversity analysis and comparative statistics. Possible functions of this marker gene analysis were predicted with PICRUSt [44] according to the tutorial (http://picrust.github.io/picrust/index.html) and Galaxy modules provided by the Huttenhower lab. Core OTUs were calculated in QIIME and served as input for the network analysis. For clustering of OTUs in the network a stochastic spring-embedded algorithm was used to generate node and edge tables and calculate eweights for each OTU. The bipartite core OTU network was then visualized in Cytoscape 2.8.3 [45] with the edge-weighted spring embedded eweight layout and customized node tables for further node size (relative abundance of OTUs), color (sample color and mixtures according to Itten's color circle) and labeling adjustments (taxonomic assignment on genus level). Finally, edge width and opacity was correlated with respective eweights.

Statistical analysis: Non-metric multidimensional scaling, hierarchal clustering using the average neighbor algorithm, and multi-response-permutation-procedure (MRPP, 999 monte carlo permutations) were conducted in the R programing environment in conjunction with the vegan package [46], as was analysis of variance (ANOVA) for univariate analysis of data [46,47]. Analysis of Similarities (ANOSIM) and two-sided Pearson correlation with Fisher's z-transformation were conducted in QIIME [39].

Results and Discussion

The comparison of a controlled cleanroom (CCR) with its uncontrolled adjoining facility (UAF) revealed new insights into the potential viable microbiome exposed to strict environmental control and regular cleaning within the built environment on the level of microbial abundance and diversity.

Microbial abundance of intact cells is highly reduced in a controlled cleanroom

As indicated by results of qPCR measurements (Fig 2), an excessive number (~ 1 log) of intact microbial cells were removed by intensive cleaning procedures from the CCR (controlled cleanroom, $6.2 * 10^3$ 16S rRNA gene copies per m^2) compared to the UAF (uncontrolled adjoining facility, $7.3 * 10^4$ 16S rRNA gene copies per m^2). The small but intact microbial cell fraction was still metabolically active as determined by ATP measurements (see Fig 2; $1.4 * 10^3$ RLU (relative light unit / m^2 for CCR and $6.2 * 10^4$ RLU / m^2), although ATP counts of the overall gross contamination (determined with the handheld device to measure ATP content) were much lower in the CCR than in UAF (> 4 fold; Table A in S1 File). Analysis of variances (ANOVA) showed significant differences between sample groups (Table C–H in S1 File) for ATP ($P = 4.27*10^{-9}$), qPCR ($P = 1.66*10^{-13}$), ATP vs. qPCR ($P = 2*10^{-16}$), ATP vs. PMA ($P = 0.02$), and total (entire ATP and DNA) vs. living (intracellular ATP and DNA from intact cells; $P = 1.21*10^{-9}$) data. On the contrary a different picture was visible without PMA treatment or the removal of extracellular ATP. In this case only minor differences could be determined between CCR and UAF regarding qPCR measurements and the total microbial portion due to many amplified sequences from dead cells ($4.4 * 10^5$ for CCR and $1.6 * 10^6$ 16S rRNA gene copies for UAF per m^2). However, the total ATP content differed by ~2 logs between CCR ($2.9 * 10^3$ RLU / m^2) and UAF ($7.5 * 10^5$ RLU/m^2) samples due to a reduced metabolic activity of dead cells, lower half-life of ATP compared to DNA or the alkaline cleaning reagents (Kleenol 30, pH 12) simply removed ATP but not DNA. The ATP-assay is applicable as a universal marker of metabolic activity for viable cells, but is strongly dependent on the physiological status, size and energy metabolism of microbes, which could bias estimations for microbial abundance per se [29]. Whereas quantitative molecular measures based on nucleic acids have the advantage of targeting microbes beyond the limitations of cultivation, these methods do not differentiate between DNA molecules derived from an extracellular origin or between dead and intact cells, which could potentially lead to overestimation of microbial abundance and diversity [25]. However, PMA helps to distinguish between gene copy counts and reads obtained from dead or intact cells according to the configuration of a microbial cell structure [27,28]. By coupling PMA treatment of samples with qPCR [31], measured 16S rRNA gene copy numbers can be distinguished between all and only intact cells. Although limitations are known for multi–layered cell structures such as microbial spores and in the presence of a high osmolarity [31,48]. Nevertheless, qPCR could be brought into relation with quantitative results of the intracellular ATP content. The equation of intracellular ATP with PMA-qPCR was surprisingly balanced $P = 0.02$ (total ATP vs. qPCR P = 2*10–16, and total vs. living $P = 1.21*10^{-9}$). The proportion of disintegrated cells or free DNA was higher (99%) inside CCR than in UAF ($P = 1.66*10^{-13}$) and we speculate that the turn-over of extracellular DNA or dead microbes seemed to be much more limited in a cleanroom environment. This might have been due to decreased microbial activity which was also indicated by low levels of ATP counts in the cleanroom. Due to many controlled environmental parameters including temperature, humidity, the tolerated amount of particles, stringent and regular cleaning procedures, as well as restricted access of staff and spacecraft materials, introduced microbes have to face an extreme

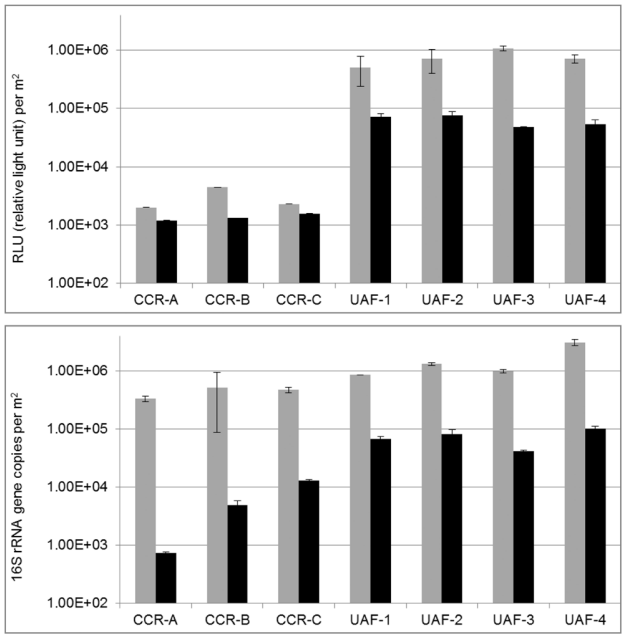

Fig 2. Quantitative evaluations. of controlled cleanroom (CCR) and uncontrolled adjoining facility (UAF) per square meter floor surface. Upper panel shows ATP counts for total (grey bars) and intracellular ATP (black bars) in CCR and UAF (total and intracellular ATP was determined in duplicate). Lower panel shows 16S rRNA gene copies in CCR and UAF with (black bars)–and without (grey bars) PMA (propidium monoazide) treatment in triplicate (error bars represent positive and negative standard deviations).

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environment, where most of them have to wait for better conditions referred to by Carrie Arnold as "waiting rooms" for microbes [13].

Microbial diversity is similar in CCR and UAF

Sequencing of the 16S rRNA gene amplicon pool on Illumina MiSeq resulted in an average yield of $2.98 * 10^4$ reads, whereas controls provided the lowest number of reads per sample and non-PMA treated samples provided the highest number of reads per sample (read statistics are summarized in Table B in S1 File). After quality filtering, chimera removal (~10%) and subtraction of reads matched with controls (13.5%; sequences with a BLAST hit of 100% were removed) [49], a range of $1.0 * 10^4$ up to $5.93 * 10^4$ quality sequences could be retrieved for further analyses.

In general microbial diversity was similar in the CCR and UAF area (average Shannon-Wiener index H': 6.6 in CCR, 6.8 in UAF), but was reduced by PMA treatment, which resulted in H': 5.8 for CCR+ and H': 6.0 for UAF+. This reduction was stable irrespective of the analyzed type of filtered data (Table I in S1 File).

The microbiome of intact cells in the controlled and uncontrolled built environment shows higher similarity than its total captured microbiome

Nonmetric multidimensional scaling (NMDS) plots based on unweighted unifrac distance matrices of rarefied OTUs to 10,011 sequences clustered CCR triplicate and samples from UAF separately (Fig 3A). CCR samples showed a higher intragroup similarity compared to UAF samples and samples treated with PMA formed more loose clusters for both rooms. Samples from the entranceway of the uncontrolled UAF facility (UAF_1) and samples from its exit

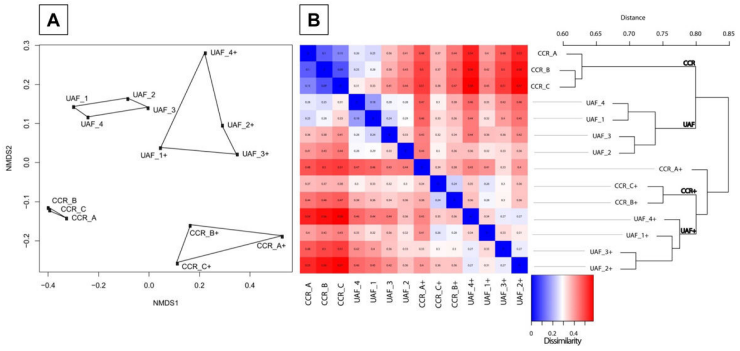

Fig 3. Beta-diversity (unweighted). (A) NMDS plot based on unweighted (left) unifrac distance matrix of rarefied OTUs to 10,011 sequences. Samples treated with PMA prior to DNA extraction are indicated by a plus symbol. CCR: controlled cleanroom. UAF: uncontrolled adjoining facility. Variances are explained per each axis (NMDS1 and NMDS2, Stress = 0.06). (B) Distance based comparison heatmap combined with a hierarchical cluster analysis based on average neighbor (HCAN) of unweighted unifrac distances. Dissimilarity of samples is indicated by a color gradient from blue (similar) via white to red (dissimilar). Samples treated with PMA prior to DNA extraction are indicated by a plus symbol. CCR: controlled cleanroom. UAF: uncontrolled adjoining facility.

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(UAF_4) into the CCR clustered closer than those samples, which were obtained from the center of the room of the uncontrolled UAF area (UAF_2 and UAF_3).

A distance-based comparison heatmap combined with an HCAN cluster dendrogram based on unweighted unifrac distances confirmed results from the NMDS analysis (Fig 3B). Similar clusters formed in both types of analysis when weighted measures were applied (S1A and S1B Fig) and if respective controls where not removed from actual samples (S2 Fig).

The grouping of samples by their respective categories (CCR, CCR+, UAF, UAF+, and controls) was significant, as determined by an ANOSIM-test R = 0.86 (P = 0.001, alpha = 0.05). Monte-carlo permutation-based analysis (MRPP) between CCR samples and UAF samples revealed a delta of 0.022 with a chance corrected within-group agreement of 0.02, indicating minor but significant differences between the sampled community structures. Even higher significant differences between PMA and non-PMA treated community structures were observed with the MRPP (delta = 0.001; chance corrected within-group agreement = 0.05). The phylogenetic composition of samples produced significant correlations using a two-sided Pearson test with Fisher's z-transformation for CCR_A-B and CCR_B-C, UAF samples 1–4 and when samples with and without their respective control were correlated.

Many dominant OTUs comprised sequences from dead cells

Microbes facing these harsh conditions were partly unique and highly diverse. In detail, the 16S rRNA gene amplicon pool contained 23 bacterial and 2 archaeal phyla in total. Three OTUs could not be classified to phylum level or a higher resolved order (one not even to a domain of life). These unclassified groups had higher abundances in UAF (1% CCR, 4% UAF) and their relative amount increased in respective samples treated with PMA (2% CCR+, 13% UAF+). Altogether, 337 OTUs could be assigned to genus level (Table J in S1 File). These sequences were dominated by *Actinobacteria* (*Corynebacterium*), *Bacteroidetes* (*Pedobacter*), *Cyanobacteria* (*Chroococcidiopsis*), *Firmicutes* (*Staphylococcus*), *Fusobacteria* (*Leptotrichia*), *Proteobacteria* (*Phenylobacterium*, *Skermanella*, *Sphingobium*, *Shewanella*, *Rickettsiella*, *Halomonas*, *Acinetobacter*, *Pseudomonas*, *Pseudoxanthomonas*), *Verrucomicrobia* (*Prosthecobacter*) and the phylum WPS-2. Twenty of these taxa were only present in CCR e.g. *Prosthecobacter*

and WPS-2, and 138 of them were only present in UAF and UAF+ (PMA treated UAF samples) e.g. *Leptotrichia*, *Skermanella*, and *Rickettsiella* (S3 Fig).

Likewise differences in PMA treated (PMA+) and non-treated samples of these dominant sequences could be observed. Similar numbers of genus level assignable OTUs appeared in PMA treated samples (33 CCR+, 38 UAF+), whereas untreated samples harbored ~ 4.6 (CCR) to ~ 6.9 (UAF) times more OTUs resolved to genus level. Overall, PMA treated samples showed sequences belonging to 70 underrepresented taxa (32 genera), which would have been masked by the predominant dead microbial species without any PMA application (Table K in S1 File). The genus *Acinetobacter*, *Pedobacter*, *Phenylobacterium*, *Sphingobium*, *Pseudoxanthomonas*, *Prosthecobacter* and the WPS-2 phylum predominantly comprised sequences from disintegrated cells, whereas sequences from *Corynebacterium*, *Chloroplasts*, *Staphylococcus*, *Skermanella*, *Shewanella*, *Rickettsiella*, *Halomonas* and *Pseudomonas* arose from intact cells (Table J in S1 File).

The microbiome of intact cells spread to a larger extent between the controlled and uncontrolled built environment

Network analysis of the core OTUs (100% present in one sample category irrespective of the other category; see Fig 4) showed different amounts of OTUs shared between CCR replicates, samples from different UAF locations, and their PMA treated fractions (CCR+ and UAF+). Whereas most OTUs of the CCR triplicate were low in abundance (indicated by respective node and font size), those OTUs shared between multiple sample categories revealed higher abundances in 16S rRNA gene amplicons e.g. *Acinetobacter* and *Pseudoxanthomonas*. However, these high abundant OTUs were composed mainly of dead cells. Core OTUs of the CCR shared only a fraction (8.2%) with core OTUs from UAF and revealed clearly which microbes

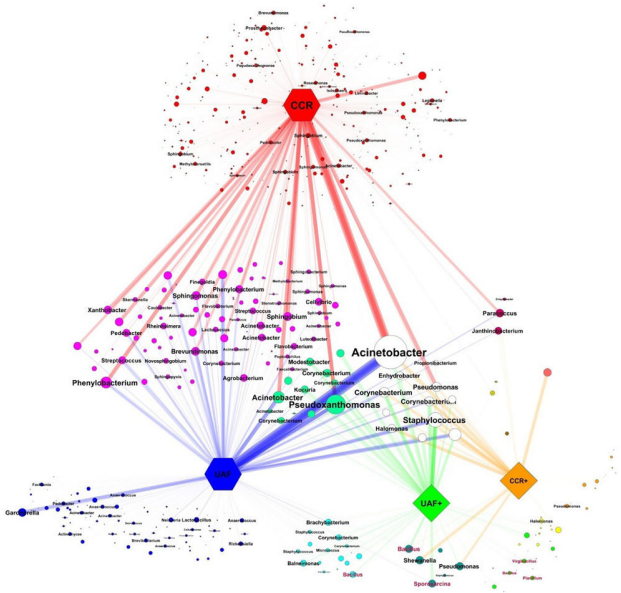

Fig 4. Core OTU network (spring embedded eweighted) of CCR (red), CCR+ (orange), UAF (blue) and UAF + (green) samples. Node size represents OTU abundance and edge width and opacity is weighted. OTUs resolved to genus level are highlighted and font size correlates with OTU abundance. Bacterial genera in red represent potential spore formers. Samples treated with PMA prior to DNA extraction are indicated by a plus symbol. CCR: controlled cleanroom. UAF: uncontrolled adjoining facility.

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were introduced from outside into the cleanroom environment (49.6%) and that the microbiome of intact cells was spread to a larger extent between both environments (CCR+: 52.4%, UAF+: 69.4%). Samples treated with PMA not only formed their own cluster in the network and ordination analysis, but also comprised most bacterial genera, which are able to form spores (e.g. *Bacillus*, *Sporosarcina*, *Virgibacillus*, *Planifilum*—highlighted in red) beside other genera. The high spreading potential of the intact microbiome may be due to the above-average proportion of survival specialists such as spore forming bacteria, which may abandon their dormant state during more suitable temporal conditions.

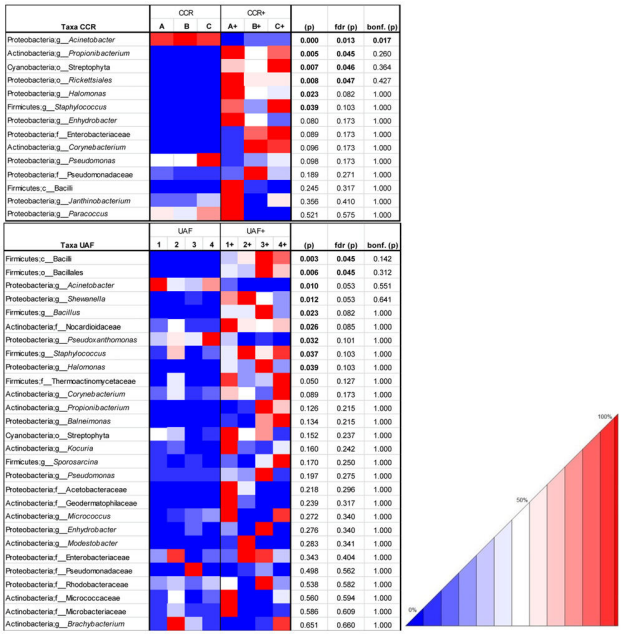
Underrepresented OTUs and taxa could be detected by the application of PMA

Some OTUs within the core microbiome showed a relative increase after being treated with PMA (see heatmaps Fig 5, S3 Fig and Table L and M in S1 File). An analysis of variance (ANOVA) showed significant increase of sequences for *Propionibacterium* (P = 0.006) and *Rickettsiales* (P = 0.008) in CCR, bacilli (P = 0.003), Bacillales (P = 0.006), *Shewanella* (P = 0.012), *Bacillus* (P = 0.023) and Nocardioidaceae (P = 0.026) in UAF, *Staphylococcus* (P = 0.039) and *Halomonas* (P = 0.039) after PMA treatment. On the contrary many dominant OTUs e.g. *Acinetobacter* (P = 0.01) and *Pseudoxanthomonas* (P = 0.032) decreased significantly after applying PMA before DNA extraction. Hence, these increasing OTUs seem to prevail as intact cells (or spores) in the core microbiome and those decreasing OTUs showed that a big part of dominant sequences arose from disintegrated cells in both environments. Notably, 11 genera (*Haloferax*, *Microlunatus*, *Virgibacillus*, *Sporosarcina*, *Balneimonas*, *Amaricoccus*, *Aquabacterium*, *Tepidimonas*, *Tolumonas*, *Shewanella*, *Rhodanobacter*) were only present in PMA treated samples from the CCR and could not be detected in the UAF of the spacecraft assembly facility.

Moreover, by its selective DNA masking properties, PMA removed dominant extracellular and DNA from compromised cells to such an extent that underrepresented taxa could be detected in the amplicon pools. The rare biosphere is not only of interest for estimating the biotechnological potential of an ecosystem [50–52], but also it is of great importance for projects related to planetary protection. By providing a highly accurate picture of microbial abundance and diversity in spacecraft assembly cleanrooms, analysis of the rare biosphere would greatly assist in assessing the risk of false positives in future live detection missions in addition to standard bioburden control protocols. Therefore, high quality thresholds for sequence filtering were set as recommended by [40], but single–and doubletons were kept in contrast to other studies, since 85% of these rare OTUs could be classified to genus or family level (Table N in S1 File).

PMA treatment helps to analyze the rare biosphere

Rarefaction analysis of all samples showed poor coverage (mean 40% ± 9) due to the immense amount of rare abundant OTUs (S4 Fig) as 99% of the microbial diversity was expressed in only 1% of the total abundance. This deep diversity analysis (less than 1% relative abundance) revealed sequences assigned to *Thaumarchaeota* (Candidatus *Nitrososphaera*), *Euryarchaeota* (*Haloferax*), *Armatimonadetes* (*Fimbriimonas*), *Chlamydiae* (Candidatus *Protochlamydia*, Candidatus *Rhabdochlamydia*), *Chlorobi*, *Chloroflexi*, *Fusobacteria* (*Fusobacterium*, *Leptotrichia*, *Sneathia*), GN02, *Gemmatimonadetes*, *Nitrospirae* (*4–29*, *Nitrospira*), *Planctomycetes* (*Gemmata*, *Isosphaera*, *A17*, *Pirellula*, *Planctomyces*), TM6, TM7, *Tenericutes* (*Mesoplasma*, *Mycoplasma*, *Ureaplasma*), *Thermi* (*Deinococcus*, *Thermus*) and *Verrucomicrobia* (*Opitutus*, *Luteolibacter*, *Prosthecobacter*, Candidatus *Xiphinematobacter*, *Ellin506*), WPS-2, and WYO. Part of this deep diversity harbored sequences from intact cells: *Thaumarchaeota*



Taxa CCR	CCR A	CCR B	CCR C	CCR+ A+	CCR+ B+	CCR+ C+	(p)	fdr (p)	bonf. (p)
Proteobacteria;g__*Acinetobacter*							**0.000**	**0.013**	**0.017**
Actinobacteria;g__*Propionibacterium*							**0.005**	**0.045**	0.260
Cyanobacteria;o__Streptophyta							**0.007**	**0.046**	0.364
Proteobacteria;o__*Rickettsiales*							**0.008**	**0.047**	0.427
Proteobacteria;g__*Halomonas*							**0.023**	0.082	1.000
Firmicutes;g__*Staphylococcus*							**0.039**	0.103	1.000
Proteobacteria;g__*Enhydrobacter*							0.080	0.173	1.000
Proteobacteria;f__Enterobacteriaceae							0.089	0.173	1.000
Actinobacteria;g__*Corynebacterium*							0.096	0.173	1.000
Proteobacteria;g__*Pseudomonas*							0.098	0.173	1.000
Proteobacteria;f__Pseudomonadaceae							0.189	0.271	1.000
Firmicutes;c__Bacilli							0.245	0.317	1.000
Proteobacteria;g__*Janthinobacterium*							0.356	0.410	1.000
Proteobacteria;g__*Paracoccus*							0.521	0.575	1.000

Taxa UAF	UAF 1	UAF 2	UAF 3	UAF 4	UAF+ 1+	UAF+ 2+	UAF+ 3+	UAF+ 4+	(p)	fdr (p)	bonf. (p)
Firmicutes;c__Bacilli									**0.003**	**0.045**	0.142
Firmicutes;o__Bacillales									**0.006**	**0.045**	0.312
Proteobacteria;g__*Acinetobacter*									**0.010**	0.053	0.551
Proteobacteria;g__*Shewanella*									**0.012**	0.053	0.641
Firmicutes;g__*Bacillus*									**0.023**	0.082	1.000
Actinobacteria;f__Nocardioidaceae									**0.026**	0.085	1.000
Proteobacteria;g__*Pseudoxanthomonas*									**0.032**	0.101	1.000
Firmicutes;g__*Staphylococcus*									**0.037**	0.103	1.000
Proteobacteria;g__*Halomonas*									**0.039**	0.103	1.000
Firmicutes;f__Thermoactinomycetaceae									0.050	0.127	1.000
Actinobacteria;g__*Corynebacterium*									0.089	0.173	1.000
Actinobacteria;g__*Propionibacterium*									0.126	0.215	1.000
Proteobacteria;g__*Balneimonas*									0.134	0.215	1.000
Cyanobacteria;o__Streptophyta									0.152	0.237	1.000
Actinobacteria;g__*Kocuria*									0.160	0.242	1.000
Firmicutes;g__*Sporosarcina*									0.170	0.250	1.000
Proteobacteria;g__*Pseudomonas*									0.197	0.275	1.000
Proteobacteria;f__Acetobacteraceae									0.218	0.296	1.000
Actinobacteria;f__Geodermatophilaceae									0.239	0.317	1.000
Actinobacteria;g__*Micrococcus*									0.272	0.340	1.000
Proteobacteria;g__*Enhydrobacter*									0.276	0.340	1.000
Actinobacteria;g__*Modestobacter*									0.283	0.341	1.000
Proteobacteria;f__Enterobacteriaceae									0.343	0.404	1.000
Proteobacteria;f__Pseudomonadaceae									0.498	0.562	1.000
Proteobacteria;f__Rhodobacteraceae									0.538	0.582	1.000
Actinobacteria;f__Micrococcaceae									0.560	0.594	1.000
Actinobacteria;f__Microbacteriaceae									0.586	0.609	1.000
Actinobacteria;g__*Brachybacterium*									0.651	0.660	1.000

Fig 5. OTU heatmap. based on taxa, which are part of the respective core microbiome from CCR (controlled cleanroom) and UAF (uncontrolled adjoining facility). Color code from blue via white to red (0–50–100%) gives relative amount [%] of respective taxonomic group. Table was rarefied to 3406 OTUs (CCR samples), and 6665 OTUs (UAF samples). Table was sorted according to resulting P-values (p) of an ANOVA test (significant (p) at an alpha of 0.05 are highlighted in bold). (p) were corrected with false discovery rate (fdr (p)) and bonferroni (bonf. (p)). Samples treated with PMA prior to DNA extraction are indicated by a plus symbol.

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(*Nitrososphaera*), *Euryarchaeota* (*Haloferax*), *Planctomycetes* (*Gemmata*, *Isosphaera*, *A17*), *Thermi* (*Deinococcus*) and *Verrucomicrobia* (*Luteolibacter*, Prosthecobacter).

These rare taxa comprised genera with potential human or plant pathogenic properties like *Afipia*, *Cupriavidus*, *Erwinia* or *Xylella*, bacterial genera involved in cycling nitrogen as *Opitutus* and *Rhodanobacter*, and archaeal halophiles–*Haloferax* and archaeal mesophiles belonging to the phylum of *Thaumarchaeota*–*Nitrososphaera*. The appearance of those taxa did not seem to arise from simple PCR bias during generation of 16S rRNA gene amplicons, since used primers have a 4-fold lower chance to cover these taxa compared to the coverage score of all detected taxa according to PrimerProspector [53] (Table O in S1 File).

Archaea and spore forming bacteria increase in terms of abundance and diversity in the microbiome of intact cells in the controlled cleanroom

As PCR bias was not an obvious explanation for this observation, the dataset was screened for trends with potential biological sources. This screening revealed a major increase of genera, which are able to form spores–*Ammoniphilus*, *Bacillus*, *Brevibacillus*, *Clostridium*, *Cohnella*, *Desulfosporosinus*, *Geobacillus*, *Paenibacillus*, *Planifilum*, *Sporosarcina*, *Terribacillus*, *Thermoactinomyces*, *Virgibacillus* and genera of the archaeal domain of life–*Haloferax* and Candidatus *Nitrososphaera*. Some of these detected genera with sequences from intact cells e.g. *Sporosarcina* or *Haloferax* were exclusively present in samples obtained from the cleanroom. The presence of these intact microbes in the microbiome of the cleanroom environment suggests that they were transferred from the gowning area into the cleanroom or even adapted themselves to these harsh environmental conditions.

Our observation that *Archaea* (*Haloferax* and Candidatus *Nitrososphaera*) occur as intact cells in a cleanroom setting, has not been previously reported. They may play an important role in microbiomes of the clean built environment, since most bacteria are either dead or have to outlast as dormant spores. *Archaea* of the phylum *Thaumarchaeota* were recently identified as an obvious contamination source in cleanrooms due to their close association to human skin [19]. Further on, the presence of intact halophiles of the archaeal domain of life in the cleanroom microbiome might arose from another human source–such as the human gut as reported by [54]. In addition we speculate that PMA introduced a shift into the amplicon population and was responsible for the increase of spore forming bacteria as this chemical cannot enter spores without treatments to increase permeability [31,55]. This shift is auxiliary for research focusing on the rare biosphere of extreme built environments and planetary protection in general (S6–S9 Figs and Table P and Q in S1 File), since sequences and taxa of great interest which may be problematic are relatively increasing not only in the aspect of abundance (archaea: 177 fold CCR, 2 fold UAF; potential spore formers: 219 fold CCR, 10 fold UAF; $P = 4^{*}10^{-4}$), but also in their respective diversity (archaea: 9 fold CCR, 3 fold UAF; potential spore formers: 3 fold CCR and UAF). Spore forming bacteria comprise most of those species which are suspected to be capable of surviving space travel and increase the risk of forward contaminations of other celestial bodies in the solar system [56]. Spore formers in general endanger food and pharmaceutical packaging in cleanrooms and pathogenic spore formers like *Bacillus anthracis* are a major threat if formulated as a bioweapon for harmful criminal interests or in the case of multi-resistant spore formers in hospitals.

Functional redundancy of PICRUSt predicted functions

The possible functional properties of taxa detected in this marker gene analysis were predicted with PICRUSt. This analysis showed a decreased dissimilarity of samples irrespective of a sample grouping per category (controlled, uncontrolled, total, viable and all, see S10 Fig and Table R in S1 File). This observation suggests that despite different microbial profiles of the CCR and UAF environment, the functional capabilities of sampled communities might be more similar than previously expected, which would lead further to a strong functional redundancy of these indoor ecosystems.

Closing remarks

Whereas microbial abundance could be reduced by strict maintenance procedures in the CCR (controlled cleanroom), microbial diversity remained almost constant compared to the UAF (uncontrolled adjoining facility, see Table 1 for a summary). In addition, the application of a PMA treatment to target the microbiome of intact cells and special groups like archaea or spore forming bacteria is very promising for other microbiome studies, since the loss of exclusive (only present with or without PMA treatment in CCR or UAF) abundance and diversity is very low.

Finally our approach also helped to identify sequences of not-yet described species from prior cleanroom studies (122 new detected genera with Illumina MiSeq compared to the following references based on 454 pyrosequencing, PhyloChip, traditional cloning and cultivation [23–26,30,57–60]. Reported genera should be considered for other clean built environments in the future, because they may serve as valuable indicator species for applications in the monitoring and validating levels of cleanliness in cleanrooms, operation theaters and hospital microbiomes.

Table 1. Summary of the microbial abundance and diversity detected in the spacecraft assembly cleanroom (controlled cleanroom–CCR) at NASA Jet Propulsion Laboratory, Pasadena, CA, USA and its surrounding uncontrolled adjoining facility—UAF. (Numbers for quantitative measures are given per m^2).

analysis	CCR	UAF
Microbial abundance		
Microbial abundance total ATP	$2.9*10^3$	$7.5*10^5$
Microbial abundance total DNA (16S rrnDB)	$4.4*10^5$	$1.6*10^6$
Viable microbial abundance		
Viable microbial abundance intracellular ATP	$1.4*10^3$	$6.2*10^4$
Viable microbial abundance PMA treated DNA	$6.2*10^3$	$7.3*10^4$
Microbial diversity (at 10,011 sequences)		
Shannon-Wiener index (H')	6.6	6.8
Phylogenic diversity (PD)	107.9	109.0
Species richness (chao 1)	5572.2	4607.1
Observed species	1673.9	1496.6
Coverage [%]	30.3	35.1
Viable microbial diversity (at 10,011 sequences)		
Shannon-Wiener index (H')	5.8	6.0
Phylogenic diversity (PD)	37.0	48.7
Species richness (chao 1)	1147.4	1635.3
Observed species	472.1	690.4
Coverage [%]	46.5	44.3
Exclusive diversity		
Exclusive microbial diversity	20 genera	138 genera
Exclusive viable microbial diversity (+ PMA)	33 genera	38 genera
Shared OTUs		
Shared core OTUs	8.2%	49.6%
Shared viable core OTUs	52.4%	69.4%
Increase in terms of abundance and diversity		
Significant increase after PMA treatment	*Propionibacterium*, *Rickettsiales*, *Staphylococcus*, *Halomonas*	*Bacilli*, *Bacillales*, *Bacillus Shewanella*, *Nocardioidaceae*, *Staphylococcus*, *Halomonas*
Abundance increase of potential spore formers by PMA treatment	219 fold	10 fold
Abundance increase of Archaea by PMA treatment	177 fold	2 fold
Diversity increase of potential spore formers by PMA treatment	3 fold	3 fold
Diversity increase of Archaea by PMA treatment	9 fold	3 fold
Exclusive taxa		
Exclusive viable genera (diversity after PMA treatment)	11	11
Exclusive genera	21	121

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Conclusion

Our room maintenance e.g. strict control of rooms, cleaning and air filtration was shown to have a large impact on microbes in the built environment. Microbial abundance can be reduced to a large extent and most microbes simply could not withstand harsh conditions and did not proliferate as indicated by low levels of intracellular ATP and DNA from intact cells. However,

the picture of microbial diversity is more complex: the diversity of microbes is only slightly reduced, since sequences of survival specialists like bacterial spore formers and archaeal halophiles and mesophiles appear to be enriched, as they are able to withstand harsh environmental conditions in the cleanroom. The bacteria detected are able to survive cleaning procedures and a low water as well as nutrient content by means of spore formation and waiting for favorable conditions. Similarly, human associated *Archaea* might be adapted to cleanroom conditions, since this domain of life is well known for their survival under many different extreme environmental conditions. Both groups of microbes have an obvious potential to interact with our human microbiome and affect our health either positively or negatively, since they are human associated and less controlled or stabilized by other species due to low amounts of intact microbes. Examinations of the ecological relevance of detected species and resolving their functions in the future should therefore aim to estimate the potential up–and downsides of strict microbial control, cleaning and reduction of microbial abundance and diversity for human health in the built environment.

Supporting Information

S1 Fig. Beta-diversity (weighted). (A) NMDS plot based on weighted unifrac distance matrix of rarefied OTUs to 10,011 sequences. Samples treated with PMA prior to DNA extraction are indicated by a plus symbol. CCR: controlled cleanroom. UAF: uncontrolled adjoining facility. Variances are explained per each axis (NMDS1 and NMDS2, Stress = 0.08). (B) HCAN based on average neighbor of weighted unifrac distances. Samples treated with PMA prior to DNA extraction are indicated by a plus symbol. CCR: controlled cleanroom. UAF: uncontrolled adjoining facility.
(TIF)

S2 Fig. PCoA plot. PCoA plot based on Bray-Curtis distance matrix. Controls of each BiSKit sampler are shown as empty grey circles. CCR: controlled cleanroom samples are shown in red and pink (+ PMA treatment), labeled A, B, C. Samples from UAF: uncontrolled adjoining facility are shown in dark and light blue (+ PMA treatment), labeled 1 to 4. Variances are explained per each axis (PC1 and PC2).
(TIF)

S3 Fig. Venn diagram. Venn diagram of detected genera in cleanroom (CCR) and gowning area (UAF). Treatment with PMA prior to DNA extraction is indicated by a plus symbol (+). Numbers indicate amount of detected genera.
(TIF)

S4 Fig. OTU heatmap. Heatmap based on taxa, which are part of the core microbiome of all samples. Color code from blue via white to red (0–50–100%) gives relative amount [%] of respective taxonomic group. Table was sorted according to resulting P-values (p) of an ANOVA test (significant (p) at an alpha of 0.05 are highlighted in bold). (p) were corrected with false discovery rate (fdr (p)) and bonferroni (bonf. (p)). Table was rarefied to 2329 OTUs. Samples treated with PMA prior to DNA extraction are indicated by a plus symbol. CCR—controlled cleanroom. UAF:- uncontrolled adjoining facility.
(TIF)

S5 Fig. Bar chart. Chart demonstrates high amount of rare abundant OTUs. 99% of the diversity is expressed in only 1% of the total number of reads. Bars in the chart are resolved to CCR (controlled cleanroom) and UAF (uncontrolled adjoining facility) respectively.
(TIF)

S6 Fig. Relative proportion of OTUs resolved to genus level. Relative proportion of OTUs grouped to potential spore forming bacteria and archaea, in presence and absence of prior PMA treatment. Panel A shows increase of OTUs assigned as spore forming bacteria after PMA treatment of samples. Panel B shows increase of OTUs assigned to archaea after PMA treatment of samples (panel B shows only one percent of the whole y-axis). Archaea P-value = 0.522; other bacteria P-value = 0.977; potential spore forming bacteria P-value = 0.000448 (ANOVA, alpha = 0.05). PMA treatment of samples is indicated by a plus symbol. CCR–controlled cleanroom, UAF–uncontrolled adjoining facility.
(TIF)

S7 Fig. Relative proportion of exclusive OTUs. Exclusive OTUs (either only present after or prior to PMA treatment) resolved to genus level and grouped to potential spore forming bacteria and archaea. Panel A shows increase of exclusive OTUs assigned as spore forming bacteria after PMA treatment of samples. Hence, PMA treatment of a sample results in more OTUs for spore formers towards a negligible quantity of OTUs getting lost by PMA treatment. Panel B shows increase of OTUs assigned to archaea after PMA treatment of samples in CCR (panel B shows only half of the whole y-axis). Archaea P-value = 0.311; other bacteria P-value = $1.15^{*}10^{-6}$; potential spore forming bacteria P-value = 0.148 (ANOVA, alpha = 0.05). PMA treatment of samples is indicated by a plus symbol. CCR–controlled cleanroom, UAF–uncontrolled adjoining facility.
(TIF)

S8 Fig. Relative proportion of microbial diversity. Microbial diversity (taxa) resolved to genus level and grouped to potential spore forming bacteria and archaea, in presence and absence of prior PMA treatment. Panel A shows increase of OTUs assigned as spore forming bacteria and archaea after PMA treatment of samples. Panel B shows magnification of panel A (panel B shows only one fourth of the whole y-axis). PMA treatment of samples is indicated by a plus symbol. CCR–controlled cleanroom, UAF–uncontrolled adjoining facility.
(TIF)

S9 Fig. Relative proportion of exclusive microbial diversity. Exclusive microbial diversity (taxa—either only present after or prior to PMA treatment) resolved to genus level and grouped to potential spore forming bacteria and archaea. Panel A shows increase of exclusive diversity assigned as spore forming bacteria and archaea after PMA treatment of samples. Hence, PMA treatment of a sample results in higher diversity for spore formers towards a negligible quantity of diversity getting lost by PMA treatment. Panel B shows magnification of panel A (panel B shows only one fourth of the whole y-axis). PMA treatment of samples is indicated by a plus symbol. CCR–controlled cleanroom, UAF–uncontrolled adjoining facility.
(TIF)

S10 Fig. 3D—PCoA plot. PCoA plot based on Bray-Curtis distance matrix of PICRUSt predicted functions from marker gene analysis. CCR: controlled cleanroom samples are shown in red and pink (+ PMA treatment). Samples from UAF: uncontrolled adjoining facility are shown in dark and light blue (+ PMA treatment). Variances are explained per each axis (PC1, PC2, and PC3).
(TIF)

S1 File. Supplementary tables. Table A: Handheld ATP counts to reveal the gross contamination of the surface to be sampled. Table B: Statistics on raw and quality filtered reads. Table C: Summary of ANOVA results on all tested categories. Table D: Summary of ANOVA results on category "ATP" with detailed results of the Tukey test. Table E: Summary of ANOVA results

on category "qPCR" with detailed results of the Tukey test. Table F: Summary of ANOVA results on categories "ATP" and "qPCR" with detailed results of the Tukey test. Table G: Summary of ANOVA results on categories "ATP" and "PMA". Table H: Summary of ANOVA results on categories "total" and "living". Table I: Microbial diversity in respect of type of the data set. Table J: OTU table of CCR and UAF samples resolved to genus level. Table K: Detected only by the use of PMA in CCR and UAF. Table L: Analysis of variances on taxa displayed in the heatmap of the core microbiome (see Fig 5). P-values were corrected for false discovery rate (fdr) and bonferroni corrected. Table M: Tukey test on ANOVA results shown in Table L. Significant P-values are highlighted in bold. Table N: Taxonomic resolution of single and doubletons till genus and family level. Table O: Coverage of primers 515f and 806r (used for Illumina MiSeq 16S rRNA gene Amplicons). Table P: Analysis of variances (ANOVA) results with Tukey test for different groups of genera (archaeal genera, potential spore forming genera and other genera). Table Q: Analysis of variances (ANOVA) results with Tukey test for different groups of exclusive (either present with or without PMA treatment in CCR or UAF) genera (archaeal genera, potential spore forming genera and other genera). Table R: Relative proportions of PICRUSt pridicted functions with its nearest sequence taxonomic index (weighted NSTI) of 16S rRNA marker gene analysis of CCR and UAF. Samples were grouped into main categories: controlled (CCR samples), uncontrolled (UAF samples), total (all untreated samples), viable (all PMA treated samples) and all (all samples).
(XLSX)

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Author Contributions

Conceived and designed the experiments: AM PV KV GB. Performed the experiments: AM PV AA. Analyzed the data: AM PV AJP. Contributed reagents/materials/analysis tools: PV AJP CME KV. Wrote the paper: AM PV AJP CME KV GB.

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Supplementary Figures to:

Cleanroom Maintenance Significantly Reduces Abundance but Not Diversity of Indoor Microbiomes

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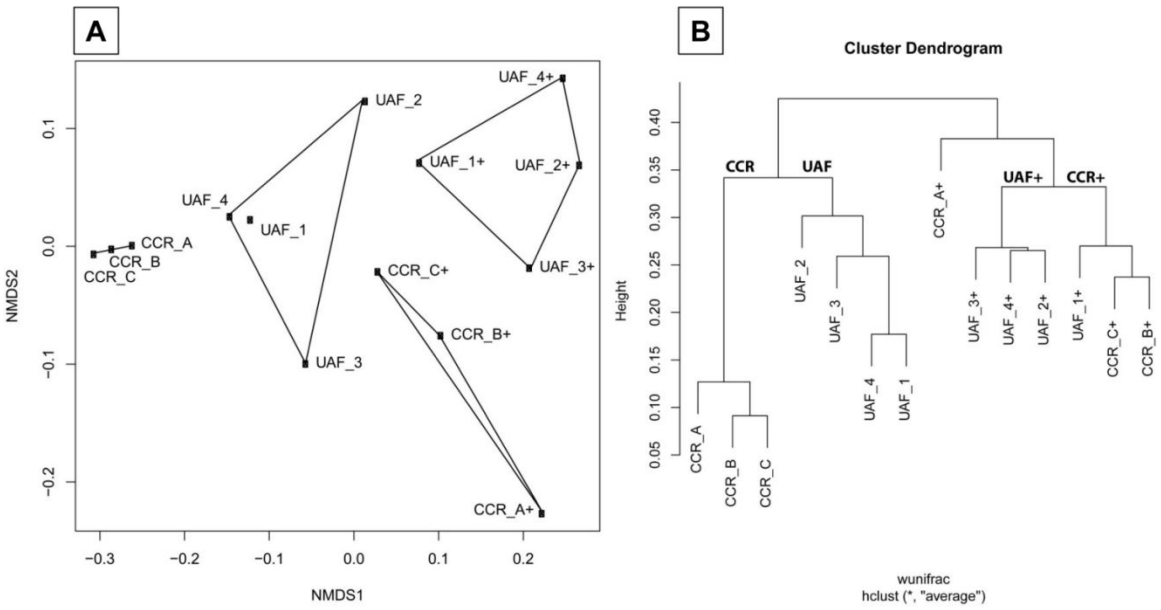
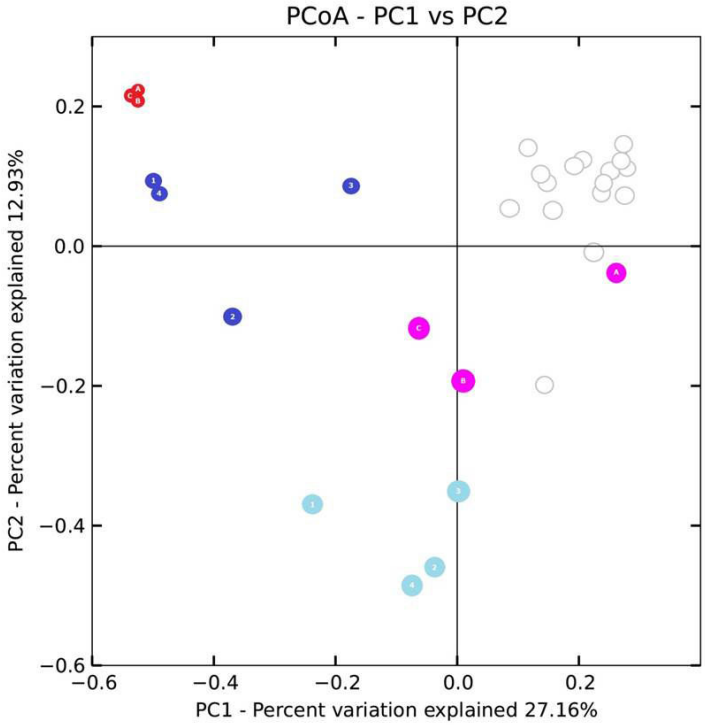
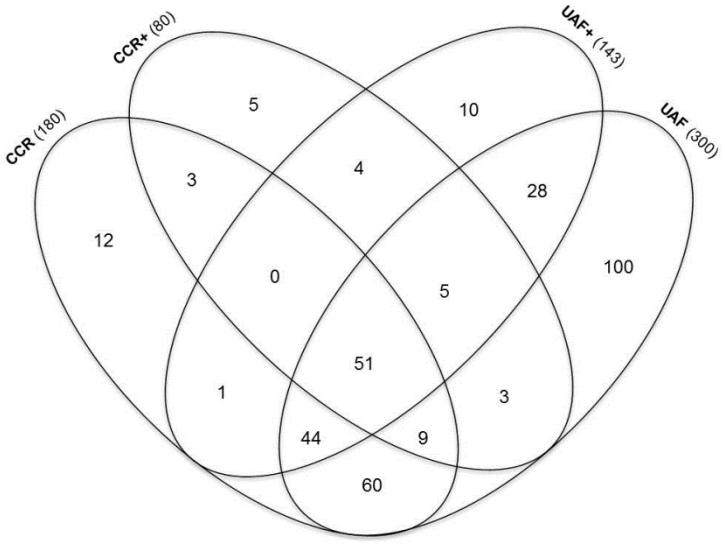
Supplementary Tables are accessible online



S1 Fig. Beta-diversity (weighted): (A) NMDS plot based on weighted unifrac distance matrix of rarefied OTUs to 10,011 sequences. Samples treated with PMA prior to DNA extraction are indicated by a plus symbol. CCR: controlled cleanroom. UAF: uncontrolled adjoining facility. Variances are explained per each axis (NMDS1 and NMDS2, Stress = 0.08). (B) HCAN based on average neighbor of weighted unifrac distances. Samples treated with PMA prior to DNA extraction are indicated by a plus symbol. CCR: controlled cleanroom. UAF: uncontrolled adjoining facility.



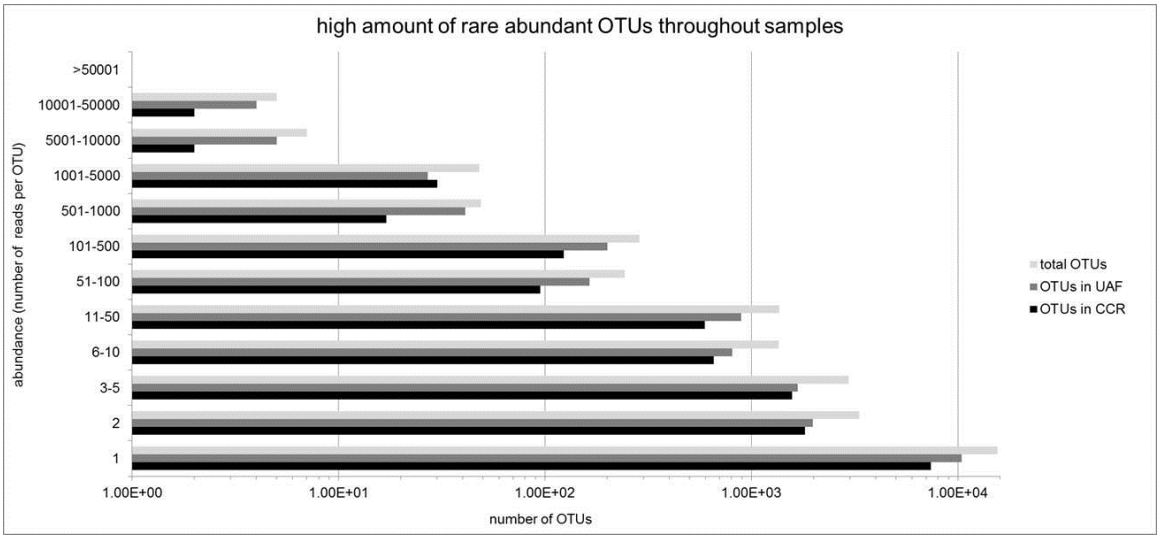
S2 Fig. PCoA plot: PCoA plot based on Bray-Curtis distance matrix. Controls of each BiSKit sampler are shown as empty grey circles. CCR: controlled cleanroom samples are shown in red and pink (+ PMA treatment), labeled A, B, C. Samples from UAF: uncontrolled adjoining facility are shown in dark and light blue (+ PMA treatment), labeled 1 to 4. Variances are explained per each axis (PC1 and PC2).



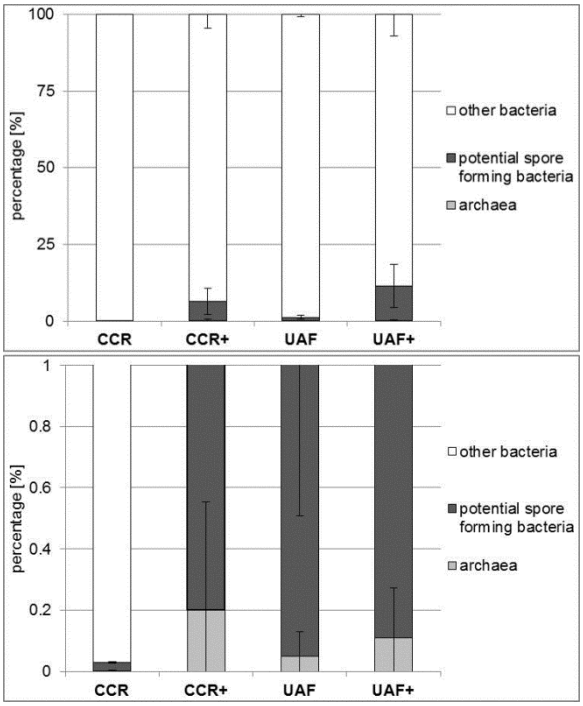
S3 Fig. Venn diagram: Venn diagram of detected genera in cleanroom (CCR) and gowning area (UAF). Treatment with PMA prior to DNA extraction is indicated by a plus symbol (+). Numbers indicate amount of detected genera.

Taxa	CCR			CCR+			UAF				UAF+				(p)	fdr (p)	bonf. (p)
	A	B	C	A+	B+	C+	1	2	3	4	1+	2+	3+	4+			
Proteobacteria;g__*Acinetobacter*															**0.00**	**0.01**	**0.03**
Proteobacteria;g__*Halomonas*															**0.00**	**0.04**	0.20
Firmicutes;g__*Staphylococcus*															**0.01**	**0.04**	0.30
Proteobacteria;g__*Pseudomonas*															**0.01**	0.05	0.64
Proteobacteria;f__Enterobacteriaceae															**0.02**	0.08	1.00
Cyanobacteria;o__Streptophyta															0.06	0.15	1.00
Actinobacteria;g__*Corynebacterium*															0.09	0.17	1.00
Actinobacteria;g__*Propionibacterium*															0.09	0.17	1.00
Proteobacteria;g__*Enhydrobacter*															0.09	0.17	1.00
Firmicutes;c__Bacilli															0.13	0.22	1.00
Proteobacteria;f__Pseudomonadaceae															0.66	0.66	1.00

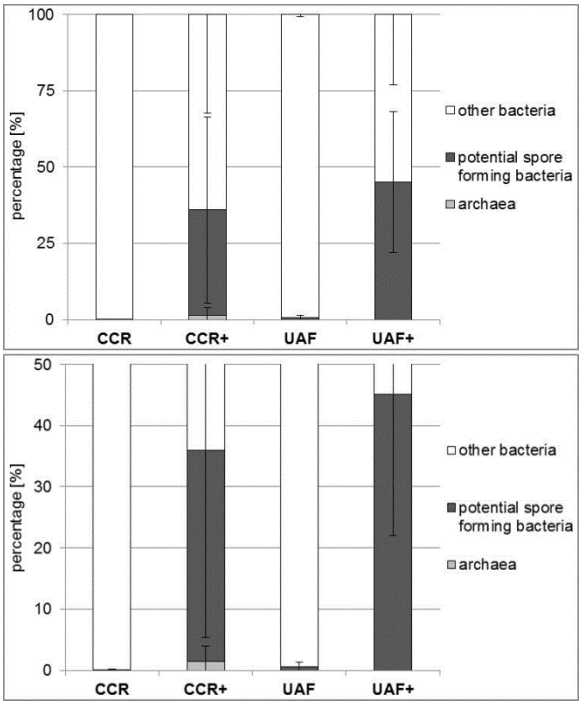
S4 Fig. OTU heatmap: Heatmap based on taxa, which are part of the core microbiome of all samples. Color code from blue via white to red (0–50–100%) gives relative amount [%] of respective taxonomic group. Table was sorted according to resulting P-values (p) of an ANOVA test (significant (p) at an alpha of 0.05 are highlighted in bold). (p) were corrected with false discovery rate (fdr (p)) and bonferroni (bonf. (p)). Table was rarefied to 2329 OTUs. Samples treated with PMA prior to DNA extraction are indicated by a plus symbol. CCR—controlled cleanroom. UAF:- uncontrolled adjoining facility.



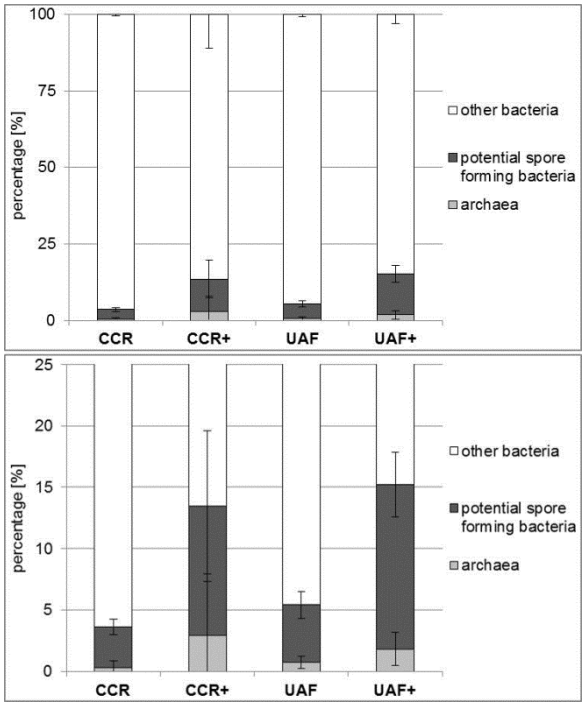
S5 Fig. Bar chart: Chart demonstrates high amount of rare abundant OTUs. 99% of the diversity is expressed in only 1% of the total number of reads. Bars in the chart are resolved to CCR (controlled cleanroom) and UAF (uncontrolled adjoining facility) respectively.



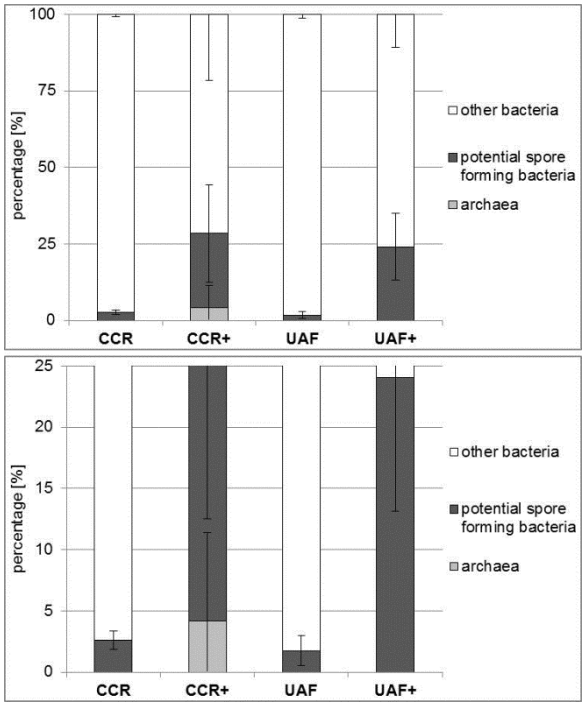
S6 Fig. Relative proportion of OTUs resolved to genus level: Relative proportion of OTUs grouped to potential spore forming bacteria and archaea, in presence and absence of prior PMA treatment. Panel A shows increase of OTUs assigned as spore forming bacteria after PMA treatment of samples. Panel B shows increase of OTUs assigned to archaea after PMA treatment of samples (panel B shows only one percent of the whole y-axis). Archaea P-value = 0.522; other bacteria P-value = 0.977; potential spore forming bacteria P-value = 0.000448 (ANOVA, alpha = 0.05). PMA treatment of samples is indicated by a plus symbol. CCR–controlled cleanroom, UAF–uncontrolled adjoining facility.



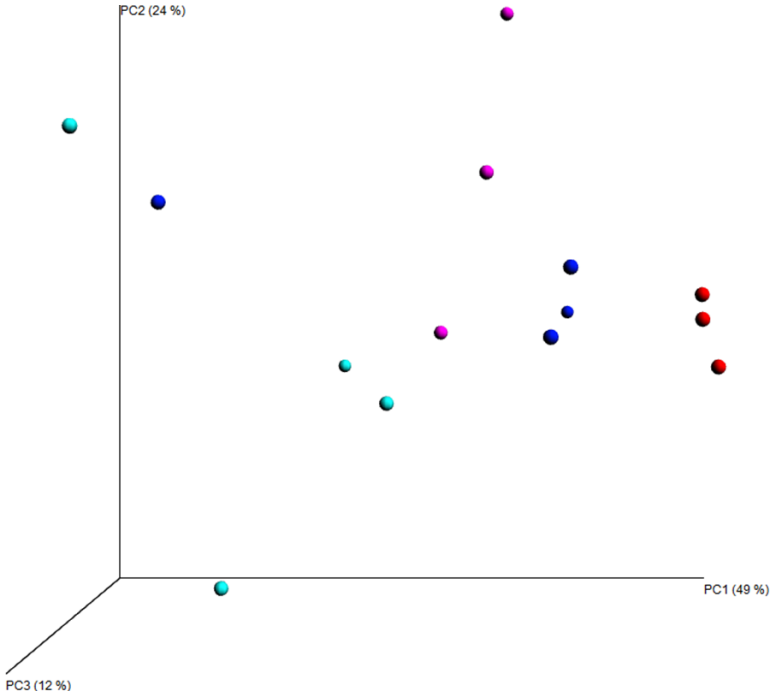
S7 Fig. Relative proportion of exclusive OTUs: Exclusive OTUs (either only present after or prior to PMA treatment) resolved to genus level and grouped to potential spore forming bacteria and archaea. Panel A shows increase of exclusive OTUs assigned as spore forming bacteria after PMA treatment of samples. Hence, PMA treatment of a sample results in more OTUs for spore formers towards a negligible quantity of OTUs getting lost by PMA treatment. Panel B shows increase of OTUs assigned to archaea after PMA treatment of samples in CCR (panel B shows only half of the whole y-axis). Archaea P-value = 0.311; other bacteria P-value = 1.15*10−6; potential spore forming bacteria P-value = 0.148 (ANOVA, alpha = 0.05). PMA treatment of samples is indicated by a plus symbol. CCR–controlled cleanroom, UAF–uncontrolled adjoining facility.



S8 Fig. Relative proportion of microbial diversity: Microbial diversity (taxa) resolved to genus level and grouped to potential spore forming bacteria and archaea, in presence and absence of prior PMA treatment. Panel A shows increase of OTUs assigned as spore forming bacteria and archaea after PMA treatment of samples. Panel B shows magnification of panel A (panel B shows only one fourth of the whole y-axis). PMA treatment of samples is indicated by a plus symbol. CCR–controlled cleanroom, UAF–uncontrolled adjoining facility.



S9 Fig. Relative proportion of exclusive microbial diversity: Exclusive microbial diversity (taxa—either only present after or prior to PMA treatment) resolved to genus level and grouped to potential spore forming bacteria and archaea. Panel A shows increase of exclusive diversity assigned as spore forming bacteria and archaea after PMA treatment of samples. Hence, PMA treatment of a sample results in higher diversity for spore formers towards a negligible quantity of diversity getting lost by PMA treatment. Panel B shows magnification of panel A (panel B shows only one fourth of the whole y-axis). PMA treatment of samples is indicated by a plus symbol. CCR–controlled cleanroom, UAF–uncontrolled adjoining facility.



S10 Fig. 3D—PCoA plot: PCoA plot based on Bray-Curtis distance matrix of PICRUSt predicted functions from marker gene analysis. CCR: controlled cleanroom samples are shown in red and pink (+ PMA treatment). Samples from UAF: uncontrolled adjoining facility are shown in dark and light blue (+ PMA treatment). Variances are explained per each axis (PC1, PC2, and PC3).

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Beneficial effects of plant-associated microbes on indoor microbiomes and human health?

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PLANT MICROBIOMES—AN INTRODUCTION

Just like humans, plants have recently been recognized as meta-organisms, possessing a distinct microbiome and revealing close symbiotic relationships with their associated microorganisms (Berg et al., 2013; Mendes et al., 2013). Each plant harbor specific species to a certain degree but also cosmopolitan and ubiquitous microbial strains; the majority of them fulfill important host as well as ecosystem functions (rev. in Berg and Smalla, 2009). In addition to the microbe-rich rhizosphere, which has been studied extensively, the phyllosphere is of special interest for the study of indoor microbiomes due to its large and exposed surface area and its remarkable microbial diversity (Lindow and Leveau, 2002; Lindow and Brandl, 2003; Redford et al., 2010; Meyer and Leveau, 2012; Vorholt, 2012; Rastogi et al., 2013). In addition to the majority of beneficial and neutral inhabitants, all plant-associated microbiomes contain plant as well as human pathogens (Berg et al., 2005; Mendes et al., 2013). A broad spectrum of plant pathogens is well-known from disease outbreaks. Human pathogens belong mainly to the so called opportunistic or facultative human pathogens such as *Burkholderia cepacia*, *Pseudomonas aeruginosa* or *Stenotrophomonas maltophilia*, which cause diseases only in patients with predisposition or in hospital (Berg et al., 2005; Ryan et al., 2009).

Microbiomes of humans and plants are currently intensively studied using the same methods and addressing similar scientific questions (Ramírez-Puebla et al., 2013). However, knowledge about the microbiomes' interaction, microbial dynamics and exchange in a certain biotope or even indoor environment is very much limited. Although the composition and function of plant microbiomes is well-studied, there is still little to no information regarding their overlap, interaction with -and impact on other microbiomes or the microbiome-harboring hosts. Information is available about the connection of soil and rhizosphere microbial diversity, which share a selective sub-set (Smalla et al., 2001). The root-soil interface is the selection site for plant-associated bacteria by root exudates, which acts as chemo-attractants as well as repellents to which bacteria respond (Badri and Vivanco, 2009). In addition, plant defense signaling play a role in this process (Doornbos et al., 2012). For the phyllosphere we know that there is only a part of residents, while a substantial part of bacteria is shared with the air microbiome (Lindow and Brandl, 2003). Based on these data, a strong interaction and exchange of rhizosphere and phyllosphere microbiomes with other microbiomes is obvious. However, this opinion paper focuses on the question, if there is also a connection from plant–to indoor microbiomes as well as an impact on human health.

INDOOR MICROBIOMES—IMPORTANCE AND ORIGIN

Despite the fact that the majority of our lifetime is spent in indoor environments such as home, work place, or public buildings, our knowledge of microbial diversity inside buildings is limited. We are not alone in these indoor environments: they provide new habitats and residence to numerous microbial communities comprising possibly hundreds of individual bacterial, archaeal and fungal species including diverse viruses. Recent studies analyzed potentially pathogenic and allergenic indoor microorganisms with mainly cultivation-based methods (Täubel et al., 2009; Yamamoto et al., 2011). Since the fraction of cultivable microbes on one specific medium is extremely low, information about specifically-adapted microorganisms, or those with special needs, remains inaccessible by standard cultivation assays. Recently, however, the application of molecular methods, including next generation sequencing (NGS) techniques has provided new insights into indoor microbial communities, revealing a generally high prokaryotic diversity including diverse bacterial, archaeal and fungal phyla (Flores et al., 2011, 2013; Moissl-Eichinger, 2011; Hewitt et al., 2012, 2013; Kembel et al., 2012; Dunn et al., 2013; Kelley and Gilbert, 2013; Meadow et al., 2013).

Indoor microbial communities are an important component of everyday human health (Arundel et al., 1986; Lee et al., 2007; Kembel et al., 2012). Due to human activity and high emission rate of up to 10^6 bacteria per person-hour as measured via 16S rRNA gene quantification from aerosols (Qian et al., 2012), indoor environments are strongly influenced by typically human-associated bacteria (Fierer et al., 2008). Hence, built environments like hospitals are more easily colonized to a large extent by patient-associated microbes (Oberauner

et al., 2013). As a result, many patients in hospitals and especially in intensive care units (ICUs) develop hospital-acquired "nosocomial infections" that compound their underlying severe disease (Vincent et al., 1995; Plowman, 2000). Moreover, these nosocomial infections remain among the leading causes of death in developed country hospitals. The risk to get nosocomial infections for patients in European ICUs was reported as 45% (Plowman, 2000). Hospital surfaces are often overlooked reservoirs for these bacteria (Hota, 2004; Gastmeier et al., 2005; Kramer et al., 2006). Apart from hospitals, indoor microorganisms affect human health as allergenic agents as well (Hanski et al., 2012). Indoor microorganisms are also involved in the development of the Sick Building Syndrome (SBS), which causes symptoms such as sensory irritation of the eyes, nose, and throat, neurotoxic or general health problems, skin irritation, non-specific hypersensitivity reactions, and odor and taste sensations (Godish, 2001).

Indoor microbiomes originate primarily from human skin, pets, or the outside air (Flores et al., 2011; Kembel et al., 2012; Meadow et al., 2013). Plants as a source of indoor microbes are so far less considered. However, air-borne microbes as substantial part—bacteria, fungi or microscopic algae—are scattered and can travel long distances such as in the wind or in clouds before returning to ground-level (Hamilton and Lenton, 1998). They have received more attention because they can serve as nuclei for condensation and as such influence our world climate as rain-making bacteria. Interestingly, cloud and hailstone studies indicated plant-surface bacteria as the dominant source of these rain-making microbes (Morris et al., 2008; Šantl-Temkiv et al., 2013). In addition, little is known about the impact of houseplants and its microbes, although older studies indicate indoor plants as important source (Burge et al., 1982).

Comparing indoor with plant microbiomes, it is our opinion that both outside and inside plants are of importance for our indoor microbiome. Plants provide beneficial bacteria for indoor rooms and therefore can positively influence human health. The following facts support our opinion about the importance of plants as source for a beneficial microbial biodiversity:

1. Empirically the positive effects of houseplants and flowers are well-known, but there is also evidence for psychological effects such as stress reduction and creative task performance (Fjeld et al., 1998; Shibata and Suzuki, 2004; Chang and Chen, 2005; Bringslimark et al., 2007; Dijkstra et al., 2008). In addition houseplants feature a remarkable capacity to improve indoor air quality (Orwell et al., 2004). This melioration of indoor air is not only due to the filtering capacity of plant leaves, but also by the degrading effects of their root associated microbes (Pegas et al., 2012 up to 90% formaldehyde removal during night according to Kim et al., 2008).
2. Plant DNA as frequently detected as chloroplast 16S rRNA gene sequences in amplicon surveys is a substantial part of all indoor microbiomes, but mainly filtered out for the presentation of data (Oberauner et al., 2013). This emphasizes, that pollen and seeds of plants, which are densely colonized by bacteria (Fürnkranz et al., 2012) are dispersed into the indoor environment and thus provide excellent shuttles for microbiome exchange.
3. Typical and often dominant plant-associated bacteria are members of the indoor microbiome. A relationship of bacteria genera occurring on plants and indoors is given in **Figure 1**. There are many ways for plant microbes to enter the built environment; as already mentioned on pollen, seeds, fog, soil on shoes, flowers, fruits and vegetables as well as transmitted by animals and other visitors.
4. At species level, no differentiation was possible for clinical and plant-associated isolates. This was studied for *Burkholderia cepacia*, *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* (Ryan et al., 2009; Martins et al., 2013). Unfortunately, these plant-associated bacteria can infect immuno-compromised patients with high predisposition in hospitals. On the one hand this is an evidence for the interplay of the plant and indoor microbiome, but on the other hand it highlights the beneficial balance, which is necessary between microorganisms and hosts.
5. Interestingly, Thaumarchaeota, originally described to be associated with ammonia-oxidation in soil and the rhizosphere of plants, have been found on human skin (Probst et al., 2013). Currently it is unknown, whether the human skin archaea have positive or negative effect on human health and whether they have different genomic capabilities compared to their soil-relatives. However, it becomes clear, that closely related microorganisms can exist in different microbiomes, based on a dynamic exchange or distribution and subsequent development of adaptation strategies.

Based on these facts, we speculate the following:

Enclosed environments and their microbiomes—like private/public buildings, hospitals, and clean rooms, which are more or less separated from outside, are especially shaped by human influence and human associated microbes (Hospodsky et al., 2012; Dunn et al., 2013). Hence, microbial diversity is altered and partially reduced compared to the outdoor environment. A reduction in microbial diversity is well known to facilitate dominant proliferations of certain strains, which might bear the risk to have a negative effect toward our health. To increase microbial diversity in an indoor environment we could simply open our windows instead of using air-condition (Hanski et al., 2012; Kembel et al., 2012; Meadow et al., 2013). Alternatively, we could use potted houseplants in built environments as a source of microbial biodiversity and possibly beneficial microorganisms.

Microbes, which live in close vicinity to human beings, are adapted to us as symbionts, commensals, or pathogens, whereas these life-styles are changeable dependent on the host-microbe balance. Indoors we share these microbes, which might get deposited on various surfaces by one person and afterwards get collected by another. Human-associated microbes e.g., skin associated, are confronted with totally new biotic and abiotic factors in the built environment. Here they have to adapt to new surface materials, compete with

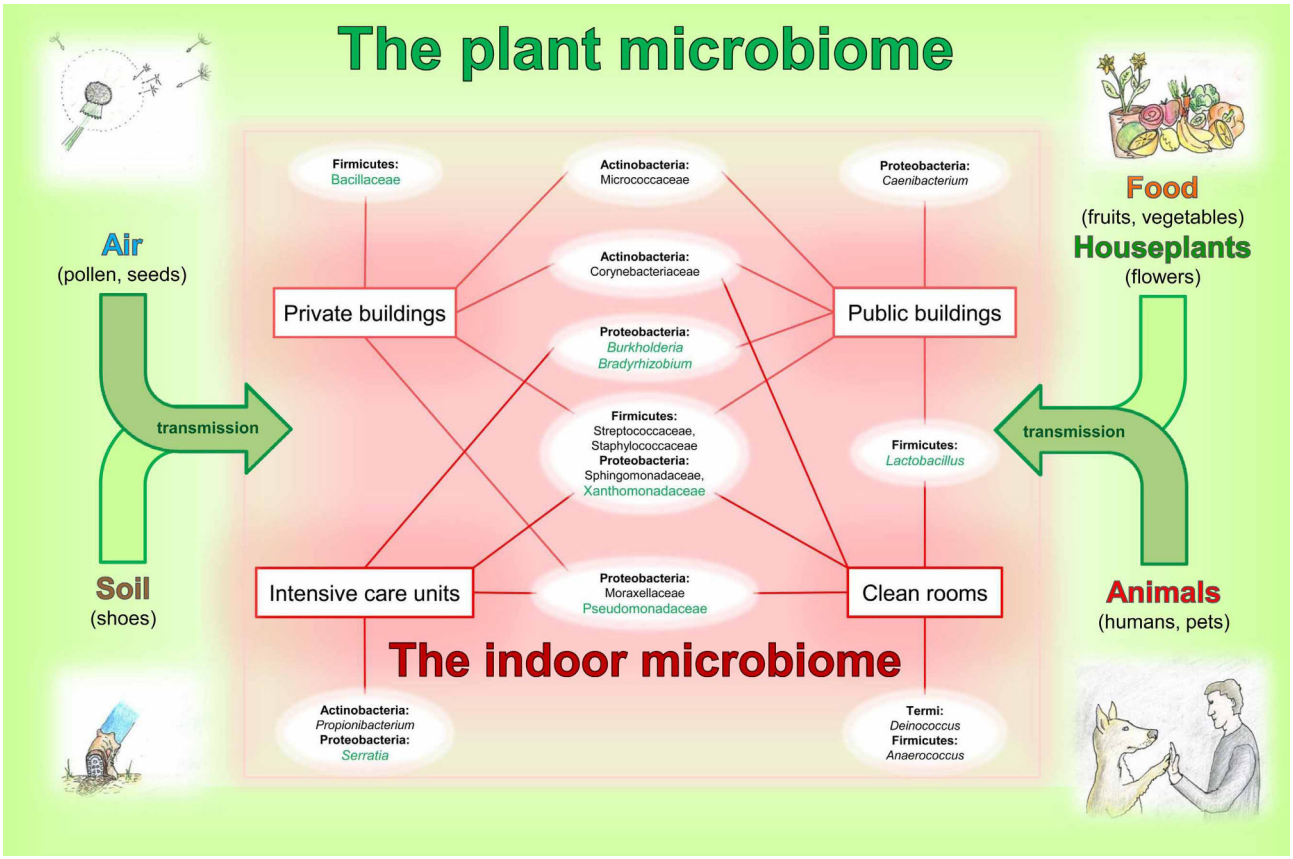

FIGURE 1 | Relationships between the plant and indoor microbiome. The indoor microbiomes, influenced by transmissions via air, soil, food, houseplants and animals from plant microbiomes, presents an overview on typical and dominant bacterial groups occurring in the built environment. Schematic chart represents occurrence of the bacterial inhabitants indoors. Bacterial families and genera (white ellipses) are arranged according to their phylum affiliation (bold) and are connected to certain types of the built environments (red squares). Taxa highlighted in green are typical phyla detectable in plant microbiomes. This image has no demand of being complete.

others for scarce nutrients and withstand stresses associated to cleaning reagents etc. However, in the case of houseplants we allow them to proliferate in a protected environment. Plant associated microbes stay on the leave or stem surface, where they have adapted to and are sheltered from cleaning procedures. Although these phyllosphere communities are confronted with an absence of direct sun light and rain as well as other changed meteorological parameters like air/dust turbulences, their rhizosphere and surrounding soil communities stay in their natural habitat. Hence, these well balanced plant communities, which we bring inside, have the potential to balance an indoor microbiome, by increasing its diversity and filter airborne microbes.

CONCLUSION

Members of the plant microbiome are an important source for indoor microbiomes. Both, plants from inside and outside can contribute to the microflora. Plant-associated bacteria could act as counterparts against pathogens within the microbial ecosystems. They stabilize the ecosystem, enhance biodiversity and avoid outbreaks of pathogens. However, more research is necessary to understand the microbiology of indoor environments. Currently used cleaning and hygiene strategies in built environments especially in hospitals and ICUs often promote multi-resistant pathogens instead of supporting beneficials. In future, it is important to re-think our understanding of necessary sterility and our relationship to our surrounding microbiomes. This "paradigm shift in ecology" is not only required for plants, humans (Jones, 2013) but also for our environment. Fortunately, "omics"-technologies guided by next-generation sequencing and microscopic techniques allow us now a much better assessment of them. Moreover, we can develop management strategies for beneficial interactions.

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Microbiome interplay: plants alter microbial abundance and diversity within the built environment

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The built indoor microbiome has importance for human health. Residents leave their microbial fingerprint but nothing is known about the transfer from plants. Our hypothesis that indoor plants contribute substantially to the microbial abundance and diversity in the built environment was experimentally confirmed as proof of principle by analyzing the microbiome of the spider plant *Chlorophytum comosum* in relation to their surroundings. The abundance of Archaea, Bacteria, and Eukaryota (fungi) increased on surrounding floor and wall surfaces within 6 months of plant isolation in a cleaned indoor environment, whereas the microbial abundance on plant leaves and indoor air remained stable. We observed a microbiome shift: the bacterial diversity on surfaces increased significantly but fungal diversity decreased. The majority of cells were intact at the time of samplings and thus most probably alive including diverse Archaea as yet unknown phyllosphere inhabitants. LEfSe and network analysis showed that most microbes were dispersed from plant leaves to the surrounding surfaces. This led to an increase of specific taxa including spore-forming fungi with potential allergic potential but also beneficial plant-associated bacteria, e.g., *Paenibacillus*. This study demonstrates for the first time that plants can alter the microbiome of a built environment, which supports the significance of plants and provides insights into the complex interplay of plants, microbiomes and human beings.

Keywords: interplay of microbiomes, indoor plants, built environment, 16S gene and ITS region amplicons, *Chlorophytum comosum*, qPCR, LEfSe analysis, network analysis

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Introduction

In recent years, deeper insight into the microbial diversity associated with plants and humans was gained using novel omics approaches; both are now recognized as meta-organisms: a functional unit of eukaryotic cells and microorganisms (Berg et al., 2014a). In contrast, the connection between microbiomes as well as the mutual exchange between them is less understood (Blaser et al., 2013). Although we live in a highly interconnected world, until the present date only a few examples of synergistic microbiomes have been discovered, which have shown that there are important relationships between single microbiomes (Berg, 2015). The rhizosphere is a well-investigated example that presents the root-soil interface influenced by the plant via root exudates as well as by the soil microbiome (Philippot et al., 2013). For instance, the rhizosphere mainly selects bacteria from soil but also contains indigenous plant-associated bacteria, e.g., bacteria derived

from seeds (Fürnkranz et al., 2012). While the rhizosphere is an example of particular importance for plant health, human health is for instance strongly dependent on the gut microbiome. David et al. (2014) recently provided evidence for the food-gut connection by analyzing the survival and metabolic activity of foodborne microbes from a plant-based diet after transit through the digestive system. Whereas this study highlighted the influence of plant-associated microbiota on the human gut microbiome, nothing is known about the impact of the phyllosphere-associated microbiota (Vorholt, 2012) on microbial abundance and diversity in the built environment. Indoor environments are considered to have big impact on human health (Reponen et al., 2012), since people in developed countries spend most of their lifetime indoors.

Built environments are not only habitats for humans; they also can be considered as biotopes for diverse microbes, whereas their abundance was mainly attributed to the presence of humans and their pets (Hanski et al., 2012; Kelley and Gilbert, 2013; Lax et al., 2014). Until now the significance of plants for humans and the built environment was mainly seen in psychological effects like mood and comfort behavior or VOCs (volatile organic compounds) as well as removal and improvement of indoor air (Sriprapat et al., 2014), but has never been linked to plant-associated microorganisms. However, is it possible that indoor plants function like humans as important or even valuable microbial dispersal sources? Our hypothesis that indoor plants contribute substantially to the microbial abundance and diversity in the built environment was already published as opinion (Berg et al., 2014b). Our hypothesis was based, amongst others, on the observation that hospital rooms that were window ventilated, contain plant-associated bacteria with potential beneficial traits for the eukaryotic hosts (Oberauner et al., 2013).

The objective of this study was to confirm our hypothesis by performing an experiment as a proof of principle, where we tracked the *Chlorophytum comosum* microbiome toward its surroundings inside an enclosed indoor environment. The spider plant *C. comosum* (Thunb.) Jacques is a monocotyledonous plant (Family *Asparagaceae*) and one of the most common indoor plants world-wide. Spider plants have been shown to have a positive impact on indoor air quality by efficiently reducing air pollution such as formaldehyde, toluene, and ethylbenzene (Sriprapat et al., 2014). Our results indicate that the plant associated microbiome spreads into the environment and might thus allow an interaction of human and plant associated microbiomes inside the built environment, which could be much more important than it had ever been assumed before.

Materials and Methods

Experimental Design

The common indoor plant *C. comosum* was kept isolated in a pre-cleaned chamber (2.27 m^3) for almost half a year. During the period of isolation the microclimate was monitored with respect to temperature and relative humidity. Samples for molecular analysis covered the indoor air (1.17 m^3), plant leaves (0.16 m^2), and surrounding surfaces (glass and press board walls and, floor tiles; 0.811 m^2) within the chamber. The plant had been part of an office inventory before it was transferred to the clean chamber. The surfaces of the chamber and all other abiotic surfaces (e.g., plant pot) were cleaned in several steps to remove microbial and DNA remnants, to be able to identify the plant's contribution to the indoor microbiome after the incubation period. First, surfaces were cleaned with water and detergents (all-purpose cleaner, Denkmit, dm-drogerie markt GmbH + Co. KG, Karlsruhe, Germany), followed by cleaning with 70% (w/v) ethanol (Carl Roth GmbH & Co KG, Karlsruhe, Germany) and Bacillol® plus (Bode Chemie GmbH, Hamburg, Germany) to remove most microbes. Chlorine bleach (DNA away, Molecular Bio Products, Inc. San Diego, CA, USA) and UV light (254 and 366 nm, Kurt Migge GmbH, Heidelberg, Germany) was used to fragment and remove remaining DNA in the chamber. The plant was placed on a pedestal in the chamber and watered once a week. Natural tab water was selected to sustain hydration of the plant. This procedure was preferred over a supply with sterilized water and soil to be more comparable with common house plants. Beside the sampling events, watering of the plant was the only period of time where the chamber was opened for some seconds and potentially susceptible to the surrounding laboratory environment. This potential input from the adjacent built environment was covered by a control (see below). Supply with light was guaranteed by natural sun light through glass windows and supported by an artificial light source according to the day/night cycle. Samples were taken in the following order: First samples from the surfaces of the cleaned chamber were received from floor and wall surfaces (surface_t0). Then samples from plant leaves were sampled before the plant was transferred to the cleaned chamber to avoid any artificial spreading of microbes due to the sampling procedure itself (plant_t0). Sampling the air (air_t0) of the chamber with the plant inside finalized all sampling steps for time point and sample group t0. Plant growth could be observed during the time of incubation. The plant was positioned on a pedestal with a reasonable distance (radius of ∼80 cm) to the surrounding wall and floor surfaces (distance of ∼36 cm to wall and floors, ∼100 cm to the ceiling). The plant had an initial volume of about 225 cm^3 and doubled its volume during the incubation period. Incubation was stopped, when the first plant leave made direct contact with the surrounding indoor surface (contact to the floor surface due to leave growth of ∼36 cm), to avoid direct transfer of phyllosphere associated microbes onto surfaces. However, throughout the incubation period, seed and flower particles were shed onto the floor surface. After the incubation period samples were taken in the following order to obtain sample group t1: First the air was sampled inside the chamber (air_t1). Then the plant was carefully removed from the chamber and the plant leaves were sampled (plant_t1). Finally surfaces of the empty chamber were sampled (surface_t1).

Sampling Procedure

Indoor air samples were obtained using the SKC BioSampler® (SKC Inc., PA, USA). All parts of the air sampler were autoclaved at 121°C for 30 min to achieve sterility and treated with dry-heat at 170°C for 24 h to degrade DNA (Probst et al., 2013). Four air sampling replications were processed in a serial manner at a flow rate of 13 l/min to allow an entire room volume to

pass through the impinger (sampling of particles from the air into PCR-grade water, Sigma-Aldrich Chemie GmbH, Stiegheim, Germany, or Carl Roth GmbH & Co KG, Karlsruhe, Germany) in about 20 min. For one replica the procedure was repeated three times (within an hour) and resulting samples (10 ml each) were pooled (30 ml total volume). For sampling plant leaves and surrounding chamber surfaces in four replications, sterile (autoclaved) and DNA-free (dry heat treatment) Alpha Wipes® (TX1009, VWR International GmbH, Vienna, Austria) were used. Alpha Wipes® were extracted in 100 ml PCR-grade water, vortexed and sonicated at 40 kHz for 2 min. Sample extracts of air, plant leave and surface samples were concentrated 100-fold to 1 ml using Amicon Ultra-15 centrifugal filter tubes (Ultracel-50K, Merck Millipore KGaA, Darmstadt, Germany). Negative controls, field blanks, sequencing controls for prokaryotes and eukaryotes and additional PMA treatment of a sample subset were processed in parallel with all samples. This procedure allowed a quality control for the sample equipment, used reagents, background signals of the indoor environment and to which extent sequences were obtained from actual intact microbial cells. Results presented in this study are based on only those samples, which passed these rigorous quality controls through PCR-testing of respective samples and controls.

PMA Treatment and DNA Extraction

PMA (propidium monoazide, GenIUL, S.L., Terrassa, Spain) treatment and DNA extraction of samples was applied as optimized and reported before (Moissl-Eichinger et al., 2015). PMA helps to determine the proportion of dead cells and free DNA in a sample, by masking free and non-membrane encased DNA in downstream processes such as PCR. Hence, after observing an over-proportional amount of intact cells compared to other enclosed indoor environments (Moissl-Eichinger et al., 2015) this procedure was not applied to all samples and represented an additional control for possible DNA contaminants and drawn conclusions of this study in general. Afterwards cells were mechanically lyzed in Lyzing Matrix E tubes filled with glass beads (MP Biomedicals, Heidelberg, Germany) on a FastPrep®-24 Instrument (MP Biomedicals, Illkirch, France) at 6.5 m/s for 2x 30 s. DNA was extracted according to the XS buffer method applicable for low biomass environments (Moissl-Eichinger, 2011).

Quantitative PCR (qPCR)

For determining microbial abundance, qPCRs with bacterial (515f—927r; 10 µM each); fungal (ITS1—ITS2; 10 µM each); and archaeal (344aF—517uR; 5 µM each) directed primers were conducted (see **Supplementary Table S1** for sequence of primers). The qPCR reaction mix for bacteria and fungi (7.04 µl) contained 5 µl QuantiTect SYBR® Green PCR kit (QIAGEN GmbH, Hilden, Germany), 0.2 µl BSA, 0.12 µl forward and reverse primers, 0.8 µl PCR grade water and 0.8 µl of the extracted genomic DNA as a template. For archaea targeted qPCR, the reaction mix (10 µl) comprised 3 µl PCR grade water, 5 µl QuantiTect SYBR® Green PCR kit (QIAGEN GmbH, Hilden, Germany), 0.5 µl forward and reverse primers (5 µM each), and 1 µl template DNA. A modified reaction mix (7 µl) was used for e.g., plant samples with observed amplification inhibitions, which might arose from plant associated inhibitory substances. 1.06 µl PCR grade water, 3.5 µl KAPA Plant PCR buffer (KAPA3G Plant PCR Kit, Peqlab, VWR International GmbH, Erlangen, Germany), 0.42 µl forward and reverse primers, 0.056 µl of KAPA3G Plant DNA-polymerase (2.5 u/µl), 0.78 µl of SYBR® Green (4x concentrate, Invitrogen™, Eugene, OR, USA), and 0.8 µl extracted DNA template.

Amplification of DNA templates and quantification of fluorescence was achieved on a Rotor-Gene™ 6000 real- time rotary analyzer (Corbett Research, Sydney, Australia) via the following PCR programs. Bacteria: 20 s at 95°C, 15 s at 54°C and 30 s at 72°C for 40 cycles followed by a melt curve from 72 to 95°C. Fungi: 40 cycles of 30 s at 94°C, 35 s at 58°C, 40 s at 72°C was used, and concluded with a melt curve. For archaea, 40 cycles of 15 s at 94°C, 30 s at 60°C, 30 s at 72°C was used followed by a melt curve. Ten individual qPCR runs with a mean reaction efficiency of 90% and R^2 values of standard curves of 0.94 were performed separately and measured in triplicate. Occasional gene copy numbers found in negative controls were subtracted from their respective samples.

Preparation of 16S rRNA Gene and ITS Region Amplicons

Amplicons were prepared with two different barcoded primer combinations: 520f—802r specific for bacteria and ITS1f—ITS2rP regions specific for fungi (see **Supplementary Table S1** for sequence of primers). Due to scattered PCR inhibitions (e.g., plant samples) for some samples Taq&Go™ Mastermix (MP Biomedicals, Heidelberg, Germany) was substituted with KAPA3G Plant PCR Kit and nested PCR procedures were applied to add barcoded primers. 1 µl template DNA was amplified on a Whatman Biometra® Tpersonal and Tgradient thermocycler (Biometra GmbH, Göttingen, Germany) and a TECHNE TC-PLUS gradient thermocycler (Bibby Scientific Ltd, Stone, UK) with the following cycling conditions: initial denaturation 95°C 5 min, denaturation 95°C 50 s, annealing 60°C 30 s (62°C 35 s for ITS regions), extension 72°C 60 s (40 s for ITS1-2). Four individual PCR reactions à 30 µl (6 µl Taq&Go™ polymerase, 18 µl PCR grade water, 1.5 µl forward and reverse primer (5 µM), 1 µl template DNA) or 50 µl (17.6 µl PCR grade water, 25 µl KAPA3G Plant PCR buffer, 0.4 µl KAPA3G Plant DNA-polymerase (2.5 u/µl), 3 µl forward and reverse primer (5 µM) and 1 µl template DNA) were pooled and transferred on a DNA free 96 well plate. The following pre-sequencing preparations were conducted by Eurofins Genomics GmbH, Ebersberg, Germany. According to HT DNA-QC (Agilent Technologies Sales & Services GmbH & Co.KG, Waldbronn, Germany) samples were pooled in equimolar concentrations in 2 pools (Pool_Bac520_Gelex and the Pool_Fungi_Gelex with 24 barcoded samples each). Library pools were provided with 2 different adaptor versions to increase complexity of samples. After quality control libraries were purified via gel extraction, quantified, and mixed. Sequencing was achieved on an Illumina MiSeq instrument with chemistry version 3 (2 × 300 bp).

Reads were filtered and sorted according to inline barcodes and individual sequencing tags. Raw reads were deposited in the European Nucleotide Archive (www.ebi.ac.uk) under project PRJEB8807 (ERP009846).

Bioinformatics and Statistics

Filtered and sorted reads were additionally length- (200–400 bp) and quality filtered (phred q20) in QIIME (Caporaso et al., 2010). Chimeric sequences were identified and removed with usearch (Edgar, 2010) using either Greengenes gg_13_8 for 16S rRNA gene reads or UNITE ver6_99_s_04.07.2014 for ITS region amplicons as a reference. OTUs (operational taxonomic units) were picked according to the open reference given above and any sequence not present in the respective reference was clustered denovo with usearch (according to 16S analysis tutorial in QIIME) and uclust for ITS reads (according to the Fungal ITS analysis tutorial in QIIME). After OTU picking, representative sequence alignment, taxonomy assignment, and tree construction, an OTU table with all metadata was generated. The rarefied OTU tables (520f—802r 4062 sequences; ITS1f—ITS2rP: 6839 sequences) served as the main input for following alpha and beta-diversity analysis. Core OTUs at 100% were calculated for each category (air_t0, air_t1, plant_t0, plant_t1, surface_t0, surface_t1) and served as input for network analysis (see Moissl-Eichinger et al., 2015 for more details) and LEfSe analysis (Segata et al., 2011) calculated with Galaxy modules provided by the Huttenhower lab. Adonis, ANOSIM, MRPP and mantel tests were calculated in QIIME (using the vegan package in R) with 999 permutations (R Core Team, 2014). One and Two Way ANOVA and *t*-tests were calculated in R (R Core Team, 2014) and MS Excel.

Results

Abiotic Parameters

Abiotic parameters (temperature, moisture) were constantly monitored to assess their impact on the microbial dispersal. The average temperature was 21.9 ± 3.2°C and reflects common conditions inside European buildings. A decrease of 13.4°C from 30.4°C (maximum temperature) in August to 17°C (minimum temperature) in December was observed (**Supplementary Figure S1**). Similarly, the average relative humidity showed a decrease from 66.7% (maximum) at the beginning of September to 18.7% (minimum) at the end of November with an average of 49.2 ± 9%. The day/night cycle resulted in a daily in/decrease of the average temperature from 0.3 ± 0.1 – 0.7 ± 0.6 °C (minimum 0.1°C in December to a maximum of 1.4°C in November) and 1.8 ± 1.1 – 3.4 ± 1.4% (minimum 0.1% in December to a maximum of 7.1% in September).

The Plant Increased the Microbial Abundance in its Environment

The statistically significant increase (*t*-test $P = 0.05$) of microbial abundance on surfaces (walls and floor) was visible after 6 months of plant isolation in an indoor environment (**Figure 1** and **Table 1**). The extent of increase was variable: the highest increase was determined for fungi (ITS region copies; up to 5 logs). For 16S rRNA gene copy numbers of Bacteria and Archaea an increase of up to 2 logs was detected. In contrast to the surrounding surfaces, the microbial abundance in the air and on plant leaves remained constant. An analysis of variance (ANOVA) showed significant variation of samples obtained from the indoor air, plant leaves, and surfaces for Archaea ($P = 7.9^{*}10^{-5}$), Bacteria ($P = 1.5^{*}10^{-3}$) and fungi ($P = 7.9^{*}10^{-4}$).

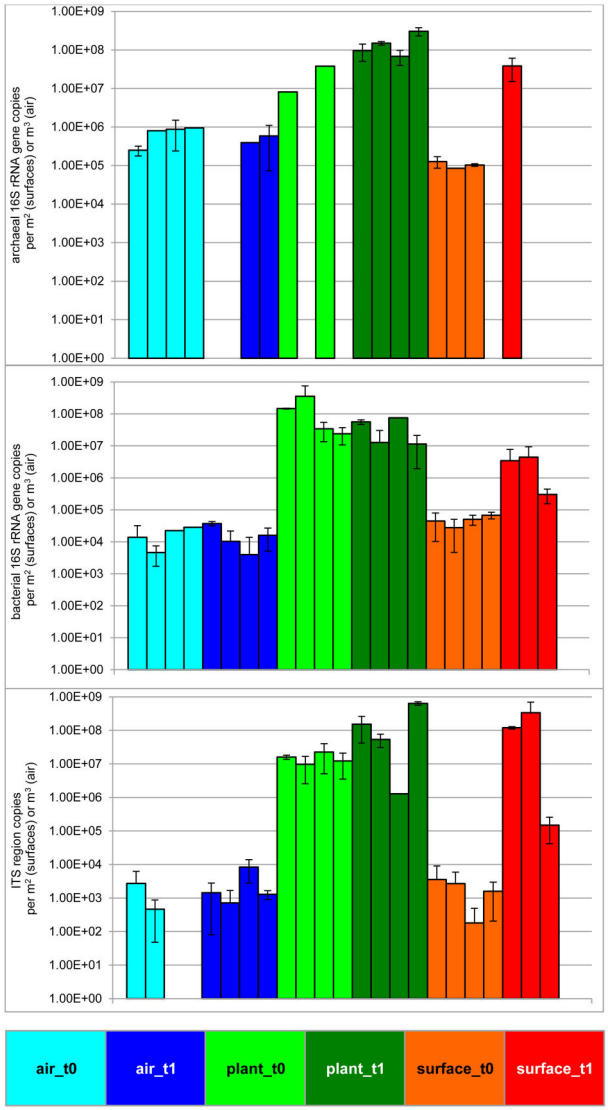


FIGURE 1 | Microbial abundance before and after plant isolation in a cleaned sealed chamber. Blue bars represent air samples, green bars—samples from plant leaves and red bars represent samples obtained from surrounding wall and floor surfaces. Brighter colors indicate the first time point (prior to isolation, t0), darker colors indicate the second time point (after plant isolation, t1) respectively. Upper panel shows results from archaeal directed 16S rRNA gene primers, middle panel gives values from bacterial 16S rRNA gene copies and lower panel presents results obtained by primers targeting the ITS region of fungi. Samples from surfaces are calculated per 1 m^2 and samples from the air are given per 1 m^3.

TABLE 1 | Summary of changes in abundance and diversity of an isolated indoor plant (*Chlorophytum comosum*) according to sampled indoor spaces (air, plant leaves, floor, and wall surfaces) and time of isolation (before plant incubation—t0, after plant incubation—t1).

	Indoor space					
	air_t0	**air_t1**	**plant_t0**	**plant_t1**	**surface_t0**	**surface_t1**
Mean archaeal qPCR copy numbers per m^2 or m^3	7.27E+05	5.39E+05	2.32E+07	1.55E+08	1.09E+05	3.83E+07
Mean bacterial qPCR copy numbers per m^2 or m^3	1.32E+04	1.98E+04	1.40E+06	3.37E+07	4.78E+04	2.38E+06
Mean fungal qPCR copy numbers per m^2 or m^3	1.91E+03	3.41E+03	1 50E+07	2.17E+08	1.09E+05	1.31E+08
Mean Shannon-Wiener index (H') bacteria	5.39	5.31	6.15	6.94	4.82	6.90
Mean Shannon-Wiener index (H') fungi	3.87	6.53	7.20	6.28	7.14	4.98
LEfSe analysis summary—taxonomic assignment of OTUs and respective LDA score (log 10)		*Acidovorax* (4.1), *Methylobacterium* (5.3)	*Cyanobacteria* (4.8)	*Saprospirae* (4.2), *Saprospirales* (4.1), *Alteromonadaceae* (4.3), *Alteromonadales* (4.3), *Bacteroidetes* (5.0), *Cellvibrio* (4.3), *Chlamydiae* (4.2), *Chlamydiales* (4.3), *Chlamydiia* (4.3), *Clostridium intestinale* (4.0), *Cytophagaceae* (4.9), *Cytophagales* (4.9), *Cytophagia* (4.9), *Devosia* (4.2), *Dyadobacter* (4.5), *Flavobacteriales* (4.0), *Flavobacteriia* (3.9), *Gemmatales* (4.3), *Gemmatimonadetes* (3.9), *Gemmatimonadetes* (3.9), *Legionellaceae* (3.9), *Legionellales* (3.9), *Luteimonas* (4.1), *Methylocystaceae* (4.2), N1423WL (3.9), *Parachlamydiaceae* (4.3), *Planctomycetes* (4.4), *Planctomycetia* (4.3), *Rhizobium* (4.1), *Sphingopyxis* (3.9), *Xanthomonadales* (4.5)	*Bradyrhizobium* (4.1), TM7 (4.1), TM7_3 (4.0)	*Acidobacteria* (4.8), *Acidobacteria_6* (4.9), *Bacilli* (4.8), *Chloroflexi* (4.0), *Paenibacillaceae* (4.2), *Rhodospirillales* (4.3), *Heterobasidion* (5.9), *Bondarzewiaceae* (5.9)

The Plant Increased the Microbial Diversity in its Environment

Microbial diversity was assessed by analyzing amplicon pools, which comprised 1,351,533 (bacteria) and 1,903,469 (fungi) quality sequences with 56,298 (bacteria) and 185,252 (fungi) picked OTUs at a 97% similarity level (**Supplementary Tables S2–S4**). The diversity changed during the time of incubation (**Table 1**). Whereas the mean bacterial diversity (calculated with the Shannon-Wiener index: H') remained almost stable on plant leaves and in the air (H' 6.15–6.94 and H' 5.39–5.31), bacterial diversity increased significantly on surrounding wall and floor surfaces (H' 4.82–6.9, *t*-test $P = 7.8^{*}10^{-33}$). On the contrary fungal diversity decreased significantly on surfaces (H' 7.14–4.98, *t*-test $P = 1.2^{*}10^{-17}$) and in the air (H' 3.87–6.53, *t*-test $P = 8.62^{*}10^{-19}$), but remained again almost stable on plant leaves (H' 7.2–6.28).

At the beta-diversity level, three distinct clusters appeared in a principal coordinate analysis based on Bray-Curtis distances of bacteria (**Figure 2A**). The first cluster was composed of samples from the air and the surrounding chamber surfaces prior to the plant isolation and the control. This cluster showed reasonable distance along PC1 axis (with a high variation of 32.6% explained) to the second cluster formed by plant leave samples prior to the isolation and the third cluster comprising samples from plant leave samples and surrounding surfaces after the isolation period. The ordination for fungi (**Figure 2B**) showed no distinct clusters of different sample groups, but similar changes in diversity along the PC1 axis (with a high variation of 22% explained). One of the most important findings was that indoor surfaces showed higher similarity to plant leaves after the isolation period. For bacteria, the calculated mean Bray-Curtis distances changed significantly (*t*-test $P = 1.7^{*}10^{-10}$) from 0.9 (surface_t0 vs. plant_t0) to 0.67 (surface_t1 vs. plant_t1) with a mean distance of all samples at 0.63. Likewise for fungi the calculated mean Bray-Curtis distances changed significantly (*t*-test $P = 2.6^{*}10^{-10}$) from 0.75 (surface_t0 vs. plant_t0) to 0.37 (surface_t1 vs. plant_t1) with a mean distance of all samples at 0.59. However, a similar trend for samples from the indoor air although less significant (*t*-test $P = 0.001$, due to a high sample dispersal) could only be perceived for the fungal communities 0.86 (air_t0 vs. surface_t0) to 0.73 (air_t1 vs. surface_t1). An adonis test (55% variation explained for bacteria and 44% for fungi) and an analysis of similarities (ANOSIM, R-statistic = 0.68 for bacteria and 0.3 for fungi) showed significant ($P = 0.001$) grouping of samples by their categories at an alpha of 0.05 with a stronger grouping per individual for bacteria. A Monte-carlo permutation based analysis (MRPP) between samples obtained from air, plant leaves, and wall and floor surfaces before and after plant isolation, resulted in a delta of 0.001 and a chance corrected within-group agreement of 0.2038 for bacteria and 0.1628 for fungi. Hence, the MRPP revealed significant differences between the overall sampled communities.

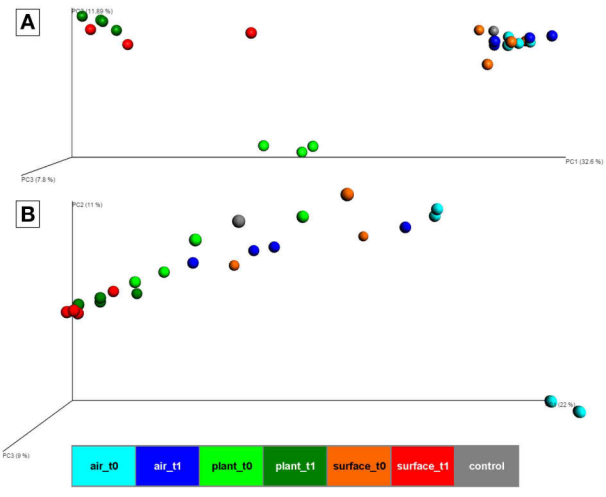


FIGURE 2 | PCoA plot with scaled coordinates by percent explained based on Bray-Curtis distances of rarefied OTU tables (4062 sequences for bacteria and 6839 sequences for fungi). (A) shows results of the bacterial 16S rRNA gene amplicons. **(B)** shows results of the fungal ITS amplicons. Spheres are colored according to the indoor space and the time points as highlighted in **Figure 1**. The control in gray was a sample from the lab environment outside the chamber after the isolation period.

LEfSe Analysis Revealed Plants as a New Source for the Microbiome within the Built Environment

The linear discriminant analysis of the effect size [LEfSe; (Segata et al., 2011)] of bacterial and fungal core OTUs revealed features that most likely explained differences between sampled indoor classes. According to this analysis 47 OTUs could be identified to be responsible for discriminating between the different sampled spaces and microbiomes (**Figure 3**). Hence, amongst other OTUs from lower taxonomic levels, *Acidovorax, Methylobacterium* (for air_t1 samples); *Caulobacter* (for control samples); *Cellvibrio, Clostridium intestinale, Devosia, Dyadobacter, Luteimonas, Rhizobium, Sphingopyxis* (for plant_t1 samples); *Bradyrhizobium* (for surface_t0 samples); and *Heterobasidion* (for surface_t1 samples) were significantly responsible to explain differences of their respective indoor space. For a deeper insight some of these OTUs are shown as abundance histograms in relation to the sampled indoor environment (**Figure 4**). This analysis showed that mainly OTUs from plant samples and surrounding floor and wall samples were significantly responsible for discriminating the different categories of indoor environments and revealed that the plant serves as a source of microbes within the built environment.

The distribution of core OTUs according to their sampled indoor spaces substantiated results obtained by the LEfSe analysis and was visualized as a core OTU network for bacteria (**Supplementary Figure S2**) and fungi (**Supplementary Figure S3**). A detailed analysis of these distribution patterns showed that most core OTUs were shared between samples from time point t1. The surrounding floor and wall surfaces were the only category where an increase from 14.1% (bacterial OTUs) and 13.5% (fungal OTUs) before plant incubation (surface_t0) to 19.8% (bacterial OTUs) and 23.1% (fungal OTUs) after plant incubation (surface_t1) could be determined. On the contrary OTUs detected in control samples

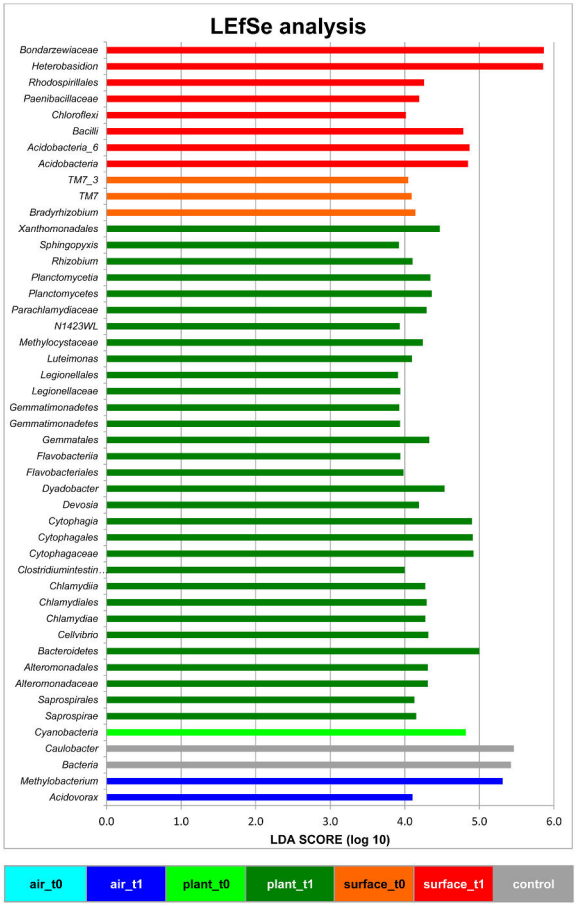


FIGURE 3 | Linear discriminant analysis Effect Size (LEfSe) of bacterial and fungal OTUs, which most likely explain differences between sampled indoor classes (indoor air, plant leaves, floor and wall surfaces prior and after plant incubation). Results were colored and grouped according to indoor classes as in **Figure 1**. The control in gray was a sample from the lab environment outside the chamber after the isolation period.

were shared to the lowest proportion (0.8% bacteria and 6.6% fungi).

The air was dominated (>10,000 sequences) by sequences assigned to *Deinococcus, Bosea genosp., Delftia, Caulobacter, Methylobacterium, Volutella, Schizophyllum commune, Trametes versicolor,* and *Aspergillus ochraceus*. The same fungal genera (the last three named genera) and species could be found to high proportions on plant leaves together with the bacterial genera *Paenibacillus, Enhydrobacter,* and *Pseudomonas*. The surfaces showed a complex mixture of these genera and species. From these taxa especially *Methylobacterium* is a common resident of the plant phyllosphere, whereas *Caulobacter* for instance is mainly associated to aquatic environments but also with phosphate-solubilizing abilities and *Delftia* is an example of a well-known genus that colonizes abiotic and biotic surfaces such as the phyllosphere. As displayed on a heatmap (**Figure 5**), many taxa were increased on the surfaces after the incubation period with the plant. A *t*-test showed for instance a significant increase for sequences of *Aspergillus ochraceus* ($P = 0.03$), *Agrobacterium* ($P = 0.03$), *Planctomyces* ($P = 0.01$), on surrounding surfaces during plant incubation. *Planctomycetes* were only recently detected since they often belong to the hitherto-uncultured bacteria (Nunes da Rocha et al., 2009). *A. ochraceus* is a soil-borne ascomycetous fungus capable of producing a variety of mycotoxins; however its airborne spores are one of the potential causes of asthma in children and lung diseases in humans.

Discussion

In the past, humans and pets were identified as important dispersal sources for microbes into the built environment. Single persons can emit up to 10^6 microbes per person and per hour (Qian et al., 2012; Dunn et al., 2013). We identified an additional effect of house plants, beyond melioration of our mood and indoor air quality, for the quality and quantity of the indoor microbiome. In a proof of principle analysis, we show in this study that plants are an additional important dispersal source in the built environment.

Our study supports our hypothesis that indoor plants contribute substantially to the microbial abundance and diversity in the built environment presented in Berg et al. (2014b) in a pilot experiment. Since plants in general influence abundance and diversity of microbes, they might be important for human wellbeing inside the built environment also from the perspective of plant-associated microbiota. Bacteria and fungi are well-known plant inhabitants, but plant-associated Archaea (*Thaumarchaeota* like *Nitrososphaera* and *Euryarchaeota* like *Halobacteriacae* and *Methanobrevibacter*) have only recently been discovered in olive leaves (Müller et al., 2015). To date, the role of Archaea in the phyllosphere is completely unknown, but their constant occurrence in many common environments might indicate basic functions in many ecosystems (Oxley et al., 2010; Bates et al., 2011; Moissl-Eichinger, 2011; Probst et al., 2013). On average 61% of detected bacterial and fungal sequences were derived from intact cells or spores as revealed by PMA (propidium monoazide) treatment of a subset of samples from all indoor spaces prior to DNA extraction, which masks DNA from compromised cells (**Supplementary Table S5**). This high rate (relative values) of intact cells from all domains of life might be due to the DNA removal and degradation procedures applied to the chamber prior to plant isolation. This uncommon, and very rigorous procedure might explain a higher proportion of intact cells compared to other indoor environments with strict cleaning procedures such as cleanrooms, with only 1% intact microorganisms, compared to 45% in garment areas (Moissl-Eichinger et al., 2015). Nevertheless, a high proportion of intact cells allow active interactions of microbes in the presence of water and nutrients, which could be tackled by metabolome studies.

The general increase of the microbial population on indoor surfaces was not surprising after such a long time of isolation in an enclosed system, but we were especially interested in identifying differences in diversity as well as sources of the microbial dispersal. Microbial diversity shifted with an increase for bacteria but a decrease for fungi on surrounding wall and

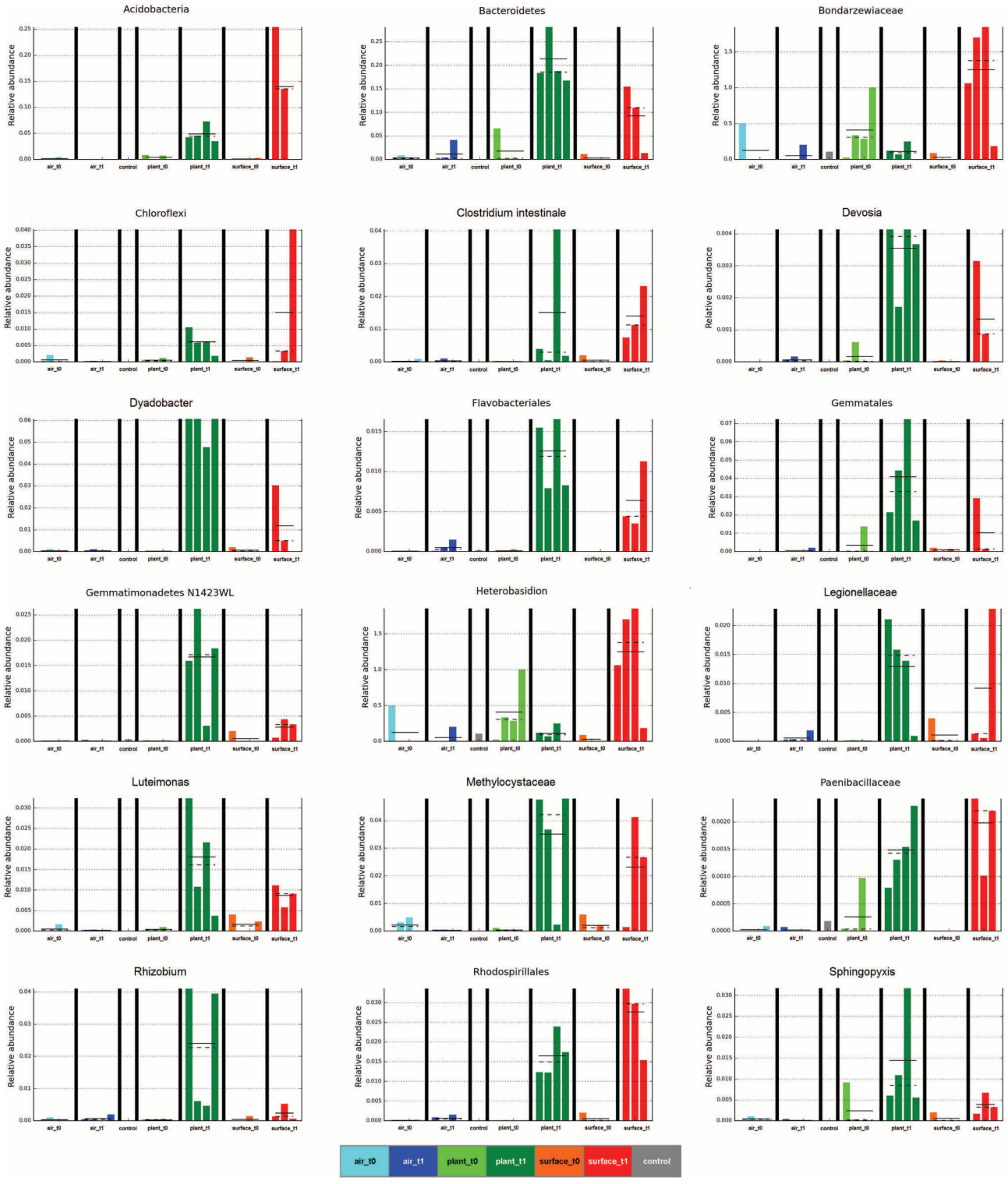
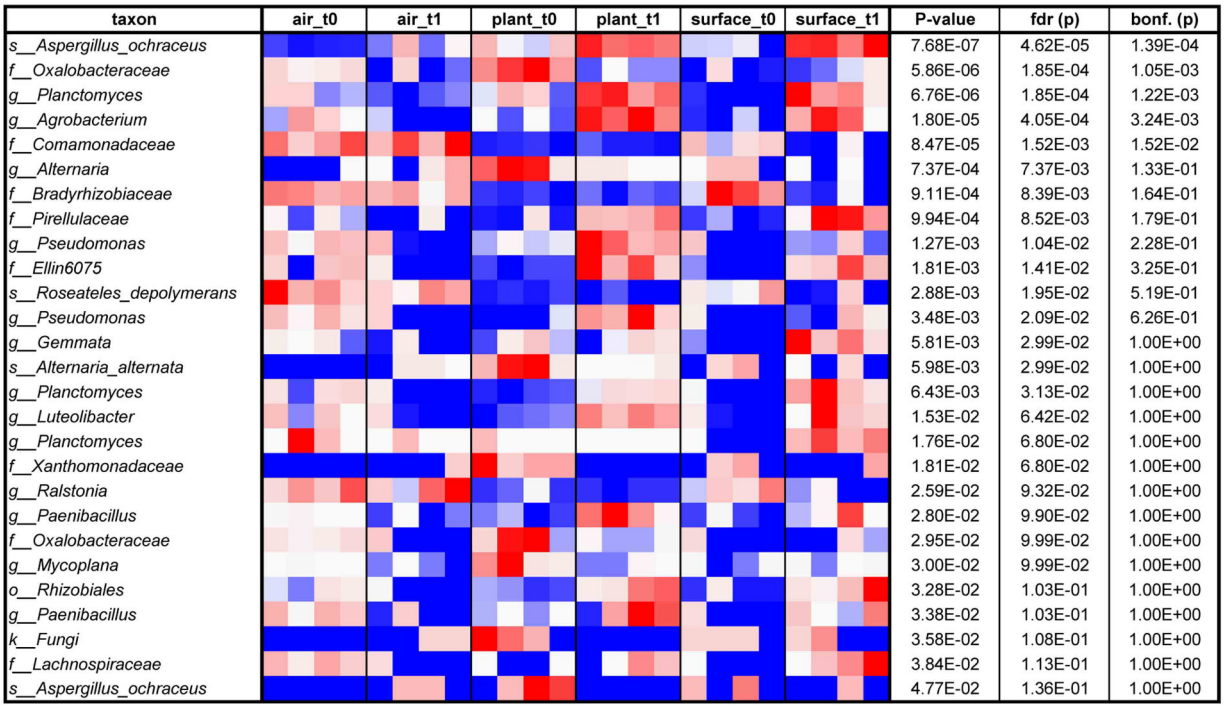


FIGURE 4 | Selected abundance histograms of features (sampled indoor spaces—indoor air, plant leaves, floor and wall surfaces prior and after plant incubation) detected by LEfSe as biomarkers. Sample groups are colored according to **Figure 1**. The control in gray was a sample from the lab environment outside the chamber after the isolation period.



taxon	air_t0	air_t1	plant_t0	plant_t1	surface_t0	surface_t1	P-value	fdr (p)	bonf. (p)
s__Aspergillus_ochraceus							7.68E-07	4.62E-05	1.39E-04
f__Oxalobacteraceae							5.86E-06	1.85E-04	1.05E-03
g__Planctomyces							6.76E-06	1.85E-04	1.22E-03
g__Agrobacterium							1.80E-05	4.05E-04	3.24E-03
f__Comamonadaceae							8.47E-05	1.52E-03	1.52E-02
g__Alternaria							7.37E-04	7.37E-03	1.33E-01
f__Bradyrhizobiaceae							9.11E-04	8.39E-03	1.64E-01
f__Pirellulaceae							9.94E-04	8.52E-03	1.79E-01
g__Pseudomonas							1.27E-03	1.04E-02	2.28E-01
f__Ellin6075							1.81E-03	1.41E-02	3.25E-01
s__Roseateles_depolymerans							2.88E-03	1.95E-02	5.19E-01
g__Pseudomonas							3.48E-03	2.09E-02	6.26E-01
g__Gemmata							5.81E-03	2.99E-02	1.00E+00
s__Alternaria_alternata							5.98E-03	2.99E-02	1.00E+00
g__Planctomyces							6.43E-03	3.13E-02	1.00E+00
g__Luteolibacter							1.53E-02	6.42E-02	1.00E+00
g__Planctomyces							1.76E-02	6.80E-02	1.00E+00
f__Xanthomonadaceae							1.81E-02	6.80E-02	1.00E+00
g__Ralstonia							2.59E-02	9.32E-02	1.00E+00
g__Paenibacillus							2.80E-02	9.90E-02	1.00E+00
f__Oxalobacteraceae							2.95E-02	9.99E-02	1.00E+00
g__Mycoplana							3.00E-02	9.99E-02	1.00E+00
o__Rhizobiales							3.28E-02	1.03E-01	1.00E+00
g__Paenibacillus							3.38E-02	1.03E-01	1.00E+00
k__Fungi							3.58E-02	1.08E-01	1.00E+00
f__Lachnospiraceae							3.84E-02	1.13E-01	1.00E+00
s__Aspergillus_ochraceus							4.77E-02	1.36E-01	1.00E+00

FIGURE 5 | Heatmap from blue (low) via white to red (high) of those taxa, which relatively increased on different indoor spaces (indoor air, plant leaves or wall and floor surfaces) sorted according to *P*-value (at an alpha of 0.05 determined by an ANOVA; in addition false discovery rate (fdr) and Bonferroni corrected *P*-values (bonf.) are shown as well).

floor surfaces as well as plant leaves. This transition could be due to unknown plant properties, but more obvious they might be the result of the altered microclimate inside the chamber after half a year of incubation (**Supplementary Figure S1**). Hence, the decrease in relative humidity might explain the lowered diversity for fungi on surfaces over time. An increasing microbial diversity on surfaces as well as the higher similarity to plant leaves could be of importance, since microbial diversity was shown to determine the invasion by a bacterial pathogen (Van Elsas et al., 2012). Due to the fact that several microbial indoor pathogens are known to be able to cause severe health problems (Nunes da Rocha et al., 2009), a higher diversity could help to avoid settling of these pathogens. LEfSe and partly the network analysis (**Figures 3**, **4** and **Supplementary Figures S2**, **S3**) revealed the importance of phyllosphere associated microbiota for the transfer of microbes and the general increase of abundance and diversity on the surrounding wall and floor surfaces. This shows that all microenvironments share a part of the microbiome and that house plants act as a bio-resource.

Altogether, plant incubation led to an increase of beneficial plant-associated bacteria *Paenibacillus* (Rybakova et al., 2015), plant-associated *Plantomycetes* with unknown function (Nunes da Rocha et al., 2009) and the spore-producing fungi *Aspergillus ochraceus*, *Wallemia muriae* and *Penicillium* spp. with allergenic potential (Reponen et al., 2012). The plant microbiome can be altered by the application of biological control agents or stress protection agents (Yang et al., 2009; Berg et al., 2013). This opportunity can be used to develop control agents with beneficial effects to plants as well as to humans. In this context it should also be possible to reduce the proportion of spore-producing fungi, since many of them harbor an allergenic potential (Reponen et al., 2012). Bacterial and fungal biocontrol agents for certain purposes have already been developed (Berg et al., 2013), but the potential of Archaea is completely unknown. Due to the fact that none of the archaeal representatives was judged to be pathogenic so far, they may be a healthy alternative.

Although the plant was identified as major source for microorganisms in a closed cabinet, our experimental design still has several limitations, which will be discussed in detail: Firstly, the study design has some artificial components. The study setup presented here might ignore many other influences between interactions of house plants with their surrounding built environment. However, to limit potential influences and make a compromise of artificial and common environmental parameters, we decided to conduct the experiment in a closed chamber, with ordinary water supply and growing substrate. Secondly, we investigated only one house plant in one incubation system. Due to limitations to reproduce identical indoor environments we focused on one incubation system to limit divergent environmental parameters with unknown effects. As a third point, we only studied two time points. The selection of two sampling points was a compromise to guarantee a low disturbance by the invasive sampling methods. Although more sampling points would help to identify the source of microbial

dispersal, we decided against this procedure since regular sampling would disturb microbial abundance and diversity and might increase the level of potential contaminations of the chamber from outside to a critical magnitude.

Additional studies with labeled microorganisms can provide further evidence for microbial dispersal from house plants. House plants are normally grown in soil, which contain a highly diverse microbiome and can influence the environment as well as the phyllosphere as shown by Rastogi et al. (2012).

Indoor plants have the potential to influence the microbiome of the built environment similar to humans and pets. Hence, aside from determining other factors like architecture, ventilation, and room maintenance etc. the microbiome of the built environment is particularly defined by its eukaryotic habitants. The embellishment of built environments with indoor plants does not have an aesthetic relevance alone, indoor plants can act as a simple but efficient way to stabilize and increase diversity of beneficial microbes in the built environment and other enclosed systems for humans in the future such as space stations or manned space missions to successfully colonize other planets.

Author Contributions

AM: study design, performed experiments, analyzed the data, wrote the manuscript; CM: reviewed the study design and manuscript; GB: study design, wrote manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00887

Supplementary Figure S1 | Microclimate recordings of 1000 measured points of temperature and humidity in the isolation chamber from August to December 2013 (x-axis). Black line indicates temperature values in °C and gray line indicates relative humidity recordings in % (y-axis).

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Supplementary Table S1 | Sequence of primers used in this study.

Supplementary Table S2 | Summary of obtained raw and quality filtered sequences.

Supplementary Table S3 | Bacterial OTUs and taxa detected in this study according to sampled indoor spaces (unassigned reads were summarized due to file size limitations).

Supplementary Table S4 | Fungal OTUs and taxa detected in this study according to sampled indoor spaces (unassigned reads were summarized due to file size limitations).

Supplementary Table S5 | Summary of controls with counts or hits in this study.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Figures to:

Microbiome interplay: plants alter microbial abundance and diversity within the built environment.

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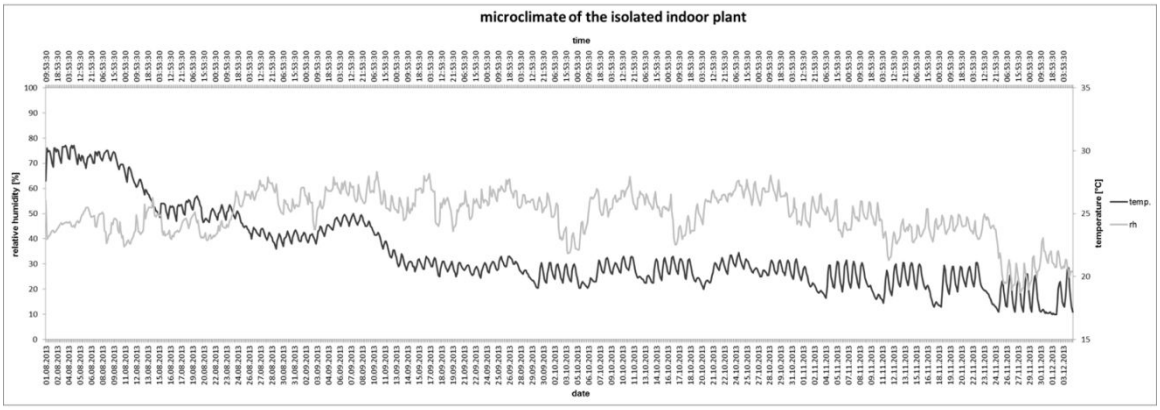
Supplementary Tables are accessible online



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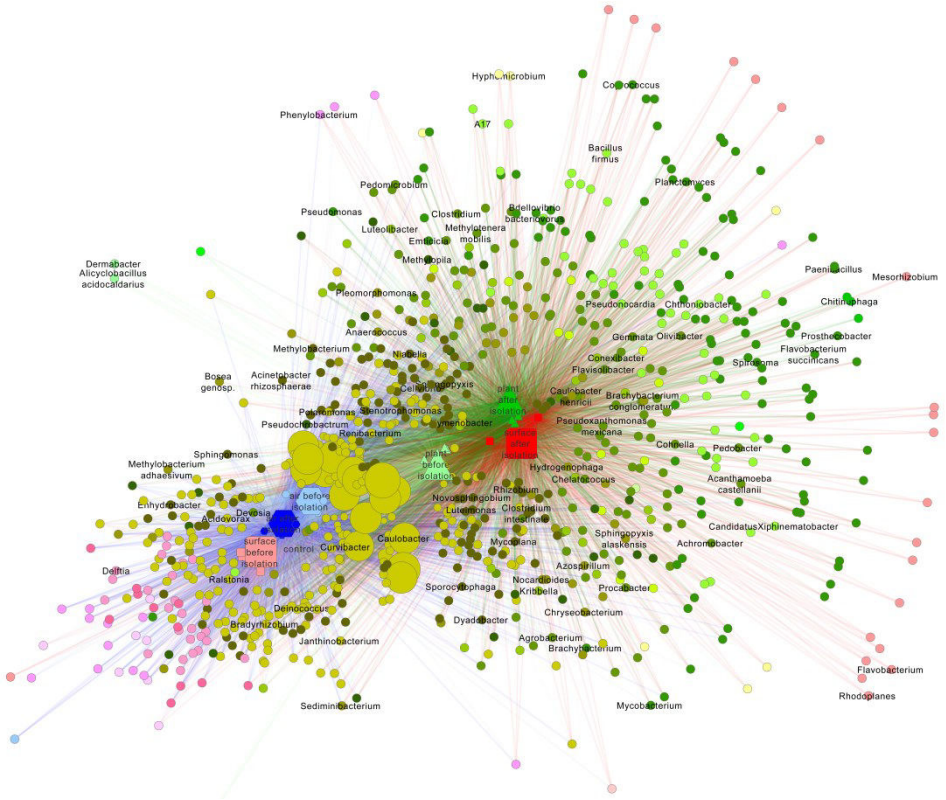

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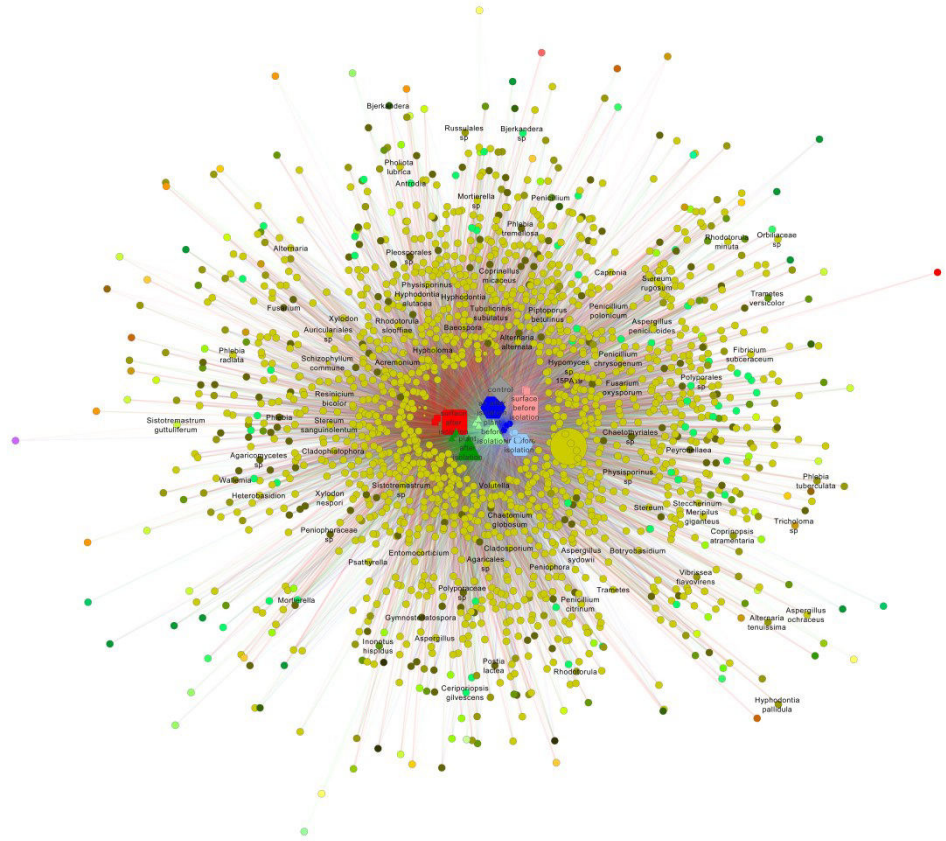

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The plant is crucial: specific composition and function of the phyllosphere microbiome of indoor ornamentals

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Abstract

The indigenous phyllosphere microbiome has been identified as a key component for plant growth, health, and for its positive effects on microbial diversity within a built environment. Nevertheless, an understanding of the phyllosphere microbiota and its driving factors remains limited. To study the variability of the microbiome in relation to plant genotype and climate, we investigated 14 phylogenetically diverse plant species grown under different controlled conditions in the greenhouses of the Botanical Garden in Graz (Austria). All investigated plants showed highly specific bacterial abundances of up to 10^6 CFU cm^{-2} on their leaves. Bacterial diversity (Shannon index H': 4.1 – 6.8) and number of putative OTUs (Chao 1: 501 – 1,097) were strongly plant species-dependent, but comprised similar dominant phyla: *Firmicutes, Proteobacteria* and *Actinobacteria.* Non-metric multidimensional scaling and BIO-ENV analysis showed a higher correlation of community composition to plant genotype in comparison to the ambient climatic variables. The antagonistic potential of the phyllosphere microbiome towards the plant pathogen *Botrytis cinerea* measured by production of antifungal volatile organic compounds (VOCs) differed in a range between 2 and 58% of the isolates. Frequently isolated VOCs producers were represented by *Bacillus ssp., Stenotrophomonas rhizophila and Kocuria ssp.* This study indicates that indoor ornamentals feature a distinct, stable microbiota with a high proportion of antifungal VOC producers on leaves irrespective of the indoor climate.

Keywords

phyllosphere microbiome, indoor ornamentals, built environments, antagonists, *Botrytis*, volatile organic compounds

Introduction

Plants harbour different microbial communities specific to each individual plant organ: the phyllosphere (Vorholt, 2012), rhizosphere (Philippot *et al*., 2013), and endosphere (Hardoim *et al*., 2015). The aboveground parts of a plant are dominated by leaves with an estimated global leaf area of 10^9 km^2 (larger than the plain surface area of our planet ~ 0.5 x 10^9 km^2, Woodward & Lomas, 2004). Although filamentous fungi, archaea, yeast, and algae are known to inhabit the leaves, bacteria are the most dominant microbial colonizers of the phyllosphere found on average of $10^6 – 10^7$ bacterial cells cm^{-2} of leaf surface (Lindow & Brandl, 2003). While the rhizosphere has been thoroughly studied for over a century, less is known about the drivers of communities in the phyllosphere (Philippot *et al*., 2013). The structure of microbial communities in the rhizosphere is influenced by soil type, but has also a strong plant species-specific component (Smalla *et al*., 2001; Berg & Smalla, 2009). This plant species-specific component depends on the plant family and its secondary metabolites, and was shown for several plant species down to the cultivar level (Cardinale *et al*., 2015). In general, leaves represent a different habitat that trigger microbial colonization, for example secondary metabolites, presence of antimicrobial wax layers, trichomes and hairs, effect a highly individual but also plant-dependent microbial composition (Bodenhausen *et al*., 2014; Ritpitakphong *et al*., 2016). Recently, carbohydrates such as sucrose, fructose and glucose, and amino acids influencing bacterial colonization were identified on the *Arabidopsis* leaf surfaces by environmental metabolomics (Ryffel *et al*., 2016). Disentangling the factors shaping microbial composition is an important objective, (Hacquard, 2016); however an overview of a broader range of plant phyla is still lacking. The phyllosphere represents the plant-air interface where the greatest impact of both biotic and abiotic conditions on the structure, diversity and function of the plant microbiome was expected to occur.

Phyllosphere colonizing bacteria are not only residents inhabiting leaves; they help to stimulate plant growth and inhibit or promote pathogen infection of plant tissues (Lindow & Brandl, 2003). Phyllosphere bacteria can additionally play a key role in carbon and nitrogen cycling (Delmotte *et al*., 2009), and help in important environmental processes such as methanol degradation (Van Aken *et al*., 2004), and nitrification (Papen *et al.*, 2002). Plants grown indoors provide specific conditions for microorganisms. For example, a distinct bacterial signature was shown for lettuce grown under greenhouse conditions and in the field (Williams & Marco, 2016), however indoor plants are hitherto less studied. Recently, beneficial effects of phyllosphere bacteria on built environments and their potential to change

microbial abundance and diversity in built environments have been reported (Mahnert *et al*., 2015). Due to the fact that we spent most of our lifetimes in built environments in many parts of the world, plants can be an important source of beneficial microbes for the human microbiome (Berg *et al.*, 2014). Despite this potential, there is limited information about the functional diversity of phyllosphere bacterial communities on the surface of the leaves of plants grown inside a built environment.

Plant-associated bacteria were recently shown to interact with host plants and other microbial species through the emissions of volatile organic compounds (VOCs), an important area of investigation that has been neglected for a long time (Ryu *et al*., 2003). VOCs are of great importance in their capacity as signaling molecules in fungal-bacterial interactions (Schmidt *et al*., 2016) and are able to suppress fungal plant pathogens (De Vrieze *et al*., 2015). However, nothing is known about how they are produced by leaf-associated bacteria and their function on indoor plants. We expect that leaf-associated bacterial strains produce VOCs to suppress plant pathogens.

The objective of our study was to analyze the phyllosphere microbiome of 14 plant species (*Aechmea eurycorymbus, Aloe arborescens, Beaucarnea recurvate, Chlorophytum comosum, Dracaena draco, Dracaena marginata, Dracaena fragrans, Epipremnum aureum, Howea forsteriana, Malvaviscus penduliflorus, Musa acuminata, Musa x paradisiaca, Nephrolepis cordifolia,* and *Olea europaea*), which represent phylogenetically different plant families as well as wide-spread indoor ornamentals. We used a unique facility, the greenhouses of the Botanical Garden of Graz, in order to study the variability of the microbiome in relation to plant genotype and climate. All of the plants in the greenhouses were grown under different controlled conditions (tropical, warm temperate, cold temperate climate), which allowed the unraveling of the factors shaping microbiota composition. We analyzed the microbiomes of leaves by using 16S rRNA gene amplicon libraries in the controlled greenhouse setting. The production of VOCs against the model pathogenic fungus *Botrytis cinerea* was additionally monitored using a novel assay for the detection of bioactive volatiles (Cernava *et al.,* 2015) in order to identify the functional potential of culturable bacterial strains.

Materials and Methods

Site description and plant maintenance inside the greenhouses

Samples were collected from the greenhouse complex of the Botanical Garden of Graz. The Botanical Garden is situated in Graz, Austria at 47°04'55" N, 15°27'28" E, with an elevation of 378 m above sea level. The greenhouse complex (Fig. S1) has four different houses simulating different terrestrial climatic conditions (Fig S2 and S3) and a nursery where all the young plants and seedlings are located. Plant-care measures for the greenhouse plants included watering, and application of fertilizers and a microbial pesticide. Watering of plants in different houses varied in frequency, depending on seasonal changes. Plants in the cold temperate house, for example, were watered only in the morning during winter, while those found in the tropical house were watered more frequently. The Botanical Garden has a cistern that catches rainwater and serves as a reservoir for watering of the plants. Two types of fertilizer were used to help maintain healthy plants: 1) is an NPK liquid fertilizer for foliar application (Wuxal® Top N), and 2) is a water-soluble Phosphate and Potash nutrient (Hakaphos® Rot 8+12+24+(4)) applied in the soil. Application of these fertilizers also varied depending on the state of plant health. The biological pesticide DiPel® was also used to protect the leaves of greenhouse plants from *Lepidoptera* larvae (caterpillar) that forages on them. This pesticide contains the naturally occurring bacteria *Bacillus thuringiensis* (Bt) *kurstaki* known for its toxicity against caterpillars. DiPel® application is carried out whenever there is an apparent infestation of Lepidopteran larvae, and is applied by spraying the solution on the leaves of affected plants. Along with foliar fertilizer and microbial pesticide, a non-ionic surfactant (Break Thru® S240) was also applied to safeguard the effectiveness of the treatments. Both protocols were taken in all greenhouse areas except the Nursery.

Sampling design and procedure

Leaves of 14 species of indoor plants were collected using sterile gloves and instruments. They were separated from the rest of the plant by cutting from the base of the petiole avoiding any possible contact with the leaf blade. Samples were placed inside 25 x 32 cm freezer bags (ARO freezer bags, Düsseldorf, Germany) immediately after collection and stored in a portable cooler with ice packs (GIO'STYLE Colombo Smart Plastics, Italy). All samples were immediately transported back to the laboratory at the Institute of Environmental Biotechnology, Graz University of Technology (TU Graz), Graz, Austria for microbial isolation and DNA extraction.

Removal of microbial cells from leaves was conducted by placing 720 cm^2 of a leaf inside a freezer bag (doubled as precaution from wear and tear) containing 50 ml 0.85% NaCl solution with Tween 80. Bags with leaves were then subjected to a series of steps including washing and sonication. Washing was done by subjecting the leaves to bag-mixer treatment (BagMixer Interscience, St. Nom, France) for 3 minutes. This step was immediately followed by sonication, using a Transsonic Digital T910 DH sonicator (Elma™, Singen, Germany), at 60 Hz for 3 min. Immediately after the first sonication step, a bag mixer treatment for 1 min, a sonication at 60 Hz for 3 min, and a final bag mixing for 1 min followed consecutively to complete the series. The resulting microbial solution was then transferred to a 50 ml Sarstedt tube. For culture-dependent experiments, 100 µl of the solution was serially diluted ten-fold and plated on both Reasoner's 2A (R2A) (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and Nutrient Broth II agar (NA) media (SIFIN, Berlin, Germany) in duplicates. The remaining microbial solution was then centrifuged (using Sorvall RC-5B Refrigerated Superspeed Centrifuge; DuPont Instruments™, USA) at 6,169 g for 20 min to pellet cells. The moist pellets were then transferred to 2.0 ml sterile Eppendorf tubes and were further centrifuged at 18,000 g for 20 minutes. Pellets were stored at −70°C until DNA extraction.

16S rRNA profiling using MiSeq Illumina Sequencing

Genomic DNA was extracted using the FastDNA® SPIN kit for soil (MP Biomedicals, Solon, OH, USA) as directed in the instruction manual. A total of 56 DNA samples were extracted; including four replicates for each of the 14 plant samples.

PCR amplifications targeting the V4 region of the 16S rRNA gene were conducted for each of the 56 samples using 515f/806r primers carrying sample-specific tags (Caporaso *et al*., 2011). Using the thermocycler TC-Plus (TECHNE, Staffordshire OSA, UK), DNA was amplified in triplicate PCR reactions (50 µl each); 25 µl of 2 x Plant buffer, 0.40 µl of 1 x KAPA3G Plant DNA polymerase, 3 µl of 5 µM for each primer, 17.60 µl PCR grade water, and 1µl of the DNA template (95°C, 3 min; 32 cycles of 95°C, 30 s; 60°C, 15 s; 72°C 12 s; and elongation at 72°C, 30s). Amplicons from three independent reactions were then pooled and purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA).

Purified amplicons were pooled in equimolar concentrations and sent for sequencing on the Illumina MiSeq platform (Eurofins Genomics, Ebersberg, Germany) with chemistry version 3 (2 x 300bp).

Bioinformatics and Statistics

Illumina reads were filtered and sorted according to barcodes on 5' and 3' prime site. Raw reads are accessible under project PRJEB13300 in the European Nucleotide Archive (www.ebi.ac.uk). Corresponding forward and reverse reads were joined, and length and quality were filtered in QIIME 1.8.0 (Caporaso et al., 2010; Mahnert et al., 2015). Barcodes and primer sequences were trimmed and chimeric sequences (reference: Greengenes gg_13_8) were removed with USEARCH (Edgar, 2010). OTUs (operational taxonomic units) were picked based on Greengenes gg_13_8 as an open reference. The resulting OTU table in biom format served as input for following alpha and beta diversity analysis and statistics. Adonis, ANOSIM (analysis of similarities), ANOVA (analysis of variances), MRPP (multi response permutation procedure), BIO-ENV (Clarke & Ainsworth 1993), and mantel tests were calculated in QIIME and R (vegan package) with 999 permutations (Dixon, 2009). Resulting BEST values of measured variables (plant species, leaf weight, temperature, and relative humidity) of the BIO-ENV were then superimposed on an NMDS (non-metric multidimensional scaling) ordination with calculated ellipses per greenhouse area.

Cultivation and isolation of bacteria

Serial dilutions of microbial solutions were plated on Reasoner's 2A (R2A) and Nutrient broth II (NB II) agar media, and incubated at room temperature for 5 days. Colony counts were expressed as CFU log10 cm^{-2} leaf. Colonies with distinct phenotypes were then transferred to 96-well plates with NB II medium and 30% glycerol for storage. Each strain was numbered according to plant sample genus (except for *Aechmea eurycorymbus* where the common name Bromelia was used as a reference), origin, and isolation medium (e.g. Dth1N1: bacterial isolate from *Draceana* from the Tropical house grown on NB II medium). All isolates were kept in at -70°C at the Institute of Environmental Biotechnology, TU Graz, Graz, Austria.

Functional characterization of isolates

A two-clamp volatile organic compounds assay (TCVA) was performed using the set-up described in Cernava *et al*. (2015) for analysis of the antagonistic property of the volatiles produced by the isolated microbes. *Botrytis cinerea*, maintained on a Potato Dextrose Agar (PDA), from the Institute of Environmental Biotechnology, TU Graz was used as model pathogen for this study. Fungal inoculum was prepared by growing the fungus for 6 days on

fresh PDA medium. After this period, the *B. cinerea* isolate was observed to have well-developed hyphae and was already sporulating.

A total of 1284 bacterial isolates were screened for their antagonistic activity against the pathogenic fungi *B. cinerea*. Isolates were streaked onto Nutrient Agar (NB II with agar) in 6-well plates and incubated for 24 h at 30°C. After the 24 h incubation period, plates observed positive for growth were clamped together with freshly prepared *B. cinerea* 6-well plates. *B. cinerea* containing plates were prepared by cutting 5 mm plugs from a 6-day old *B. cinerea* inoculum plate and placing it on the center of each well of a 6-well plate with Synthetic Nutrient-Poor Agar (SNA pH adjusted to 5.5). Setting up of the plate-pair was carried out according to Cernava *et al*. (2015) and was done in four replications. The set-up was incubated at room temperature for 3 days under dark conditions to eliminate any light-induced effect on the experiment and inhibition of growth was indicated as percentage (%).

Characterization and identification of isolates

BOX-PCR fingerprint analysis followed by Sanger sequencing was carried out in order to group the active-VOCs-producing bacterial isolates. DNA was extracted by homogenization of bacterial colonies using the MP FastPrep-24 sample preparation system FastPrep-24 Instrument (Illkirch, France) (30 s; 6 ms^{-}). Homogenized samples were frozen (at -20°C for 30 min), heat-shocked at 100°C, and immediately centrifuged (16,000 g at 4°C for 5 min; HERMLE Labor Technik, Germany). Using the Tpersonal Combi, Biometra thermocycler (Biometra GmbH, Germany) DNA was amplified in 25 µl PCR reaction mix; containing 1 µl of the extracted DNA, 5 µl of Taq&Go PCR mix, 2.50 µl of 100 pmol ml^{-} BOX A1 primer (5' CTA CGG CAA GGC GAC GCT GAC G 3'), and 16.50 µl PCR grade water (95°C, 6 min; 35 cycles of 94°C, 1 min; 53°C, 1 min, and 65°C, 8 min; with final extension at 65°C, 16 min). The resulting BOX-PCR fingerprints were evaluated using the GelComparII program (Kortrijk, Belgium). Cluster analysis was done using the unweighted pair-group average (UPGMA) algorithm.

Representative isolates from each box cluster were used for Sanger sequencing. DNA extraction was done by denaturation of isolates (preheating at 105°C; denaturation at 98.05°C, 15 min; final hold at 10°C; using the thermocycler TC-Plus (TECHNE, Staffordshire OSA, UK) immediately followed by centrifugation (3,220 g for 2 min; using Centrifuge 5810 R (Eppendorf, Germany). PCR amplification for each strain was performed using 30 µl of PCR reaction mix (6 µl Taq&Go, 1.5 µl 27f primer, 1.5 µl 1492r primer, 20 µl PCR grade water, and 1 µl DNA template). Amplification was done using a TC-Plus thermocycler (TECHNE,

Staffordshire OSA, UK) (95°C, 5 min; 40 cycles of 95°C, 30 s; 57°C, 30s; 72°C, 1 min 30 s; final extension at 72°C, 5 min). PCR products were then purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA), and sent to LGC Genomics (Berlin, Germany) for Sanger sequencing. Sequences were identified by BLASTn against the NCBI 16S rRNA gene reference database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sanger sequences with a minimum length of 700 bp were considered acceptable for analysis.

Results

Phyllosphere bacterial community structure

A total of 1.04 x 10^6 sequences were generated for the 14 plant samples. The average read number per sample was 18,594.68; ranging from 757 to 61,561 sequences. A total of 12,704 operational taxonomic units (OTUs) were identified at 97% similarity. Amplicon reads showed a high relative abundance of the phyla *Firmicutes* (40.4 %), *Proteobacteria* (22.5 %), and *Actinobacteria* (15.7 %) (Fig. 1). Two archaeal phyla were also detected, namely *Crenarcheota* and *Euryarchaeota* with 0.13 % and 0.01 % abundance, respectively.

Members of the phylum *Firmicutes* were most abundant on the phyllosphere of plants from the tropical, warm temperate, and succulent houses of the greenhouse complex, whereas *Proteobacteria* were most abundant on the phyllosphere of plants from the cold temperate house, and *Deinococcus-Thermus* in the nursery (Fig. 2A). Relative abundance of bacterial phyla also differed for each plant. *Firmicutes* were found in greatest abundance on the leaves of *Epipremnum aureum* (88.6 %), *Musa x paradisiaca* (61.4 %), *Dracaena fragrans* (74.7 %), *Howea fosteriana* (48.8 %), *Dracaena draco* (62.5 %), *Olea europaea* (40.5 %), and *Beaucarnea recurvata* (77.5 %), while *Proteobacteria* were observed in their greatest abundance on the leaves of *Malvaviscus penduliflorus* (50.6 %) and *Chlorophytum comosum* (44.0 %). On the other hand, *Actinobacteria* were found in greatest abundance on the leaves of *Dracaena marginata* (35.9 %) and *Musa acuminata* (56.0 %), and phylum *Deinococcus-Thermus* on the leaves of *Aechmea eurycorymbus* (40.7 %), *Nephrolepis cordifolia,* (44.1 %) and *Aloe arborescens* (41.8 %) as shown in Fig. 2B. Variability in the taxonomic structure of the phyllosphere community was also observed for each replicate of respective plant species (Fig. S4).

The relative abundance of sequences assigned to a genus at a cut-off of 1% is shown in Fig. 3. Highly abundant genera included *Deinococcus* (10.4%), *Arthrobacter* (4.6%), *Sphingomonas* (4.4%), *Bacillus* (4.2%), and one unidentified genus from the family *Bacillaceae* (30.1%). The unidentified genus from *Bacillaceae* was observed to be ubiquitous in all the plant samples. The abundance of *Bacillus* was found higher than 1% in all samples except on *Neprholepis cordifolia*, and *Sphingomonas* was found ≥ 1% in all samples except *Epipremnun areum* and *Beaucarnea racurvata*.

The diversity of the phyllosphere bacterial communities

Rarefaction analysis including diversity, richness and evenness estimates revealed variation in the phyllosphere bacterial communities per greenhouse and per plant sample. The rarefaction curves of phyllosphere bacterial communities from different greenhouse areas are shown in Fig. S5A. The curves showed low slopes and did not reach the saturation point. Correspondingly, the number of OTUs observed covered only 26.2 % - 29.8 % of the estimated taxonomic richness (Chao1), where putative OTUs ranged from 510.5 – 1,097.1, as seen in Table 1. The computed Shannon index of diversity (H') per greenhouse area was found highest in plants inside the cold temperate house (6.6) and was lowest in plants inside the tropical house (4.2). Statistical analysis using Two-sample T-Tests showed that diversity of bacterial communities found on the plants from the cold temperate house varied significantly from the diversity of phyllosphere bacterial communities found on the plants in the tropical (*P=0.01*) and succulent (*P=0.03*) houses. However, the diversity of bacterial communities found on plants from the nursery (*P=1.0*) and warm temperate house (*P=0.97*) did not vary significantly, compared to the diversity of phyllosphere bacterial communities of the cold temperate house. It was also observed that the diversity of the phyllosphere bacterial community in the tropical house showed significant difference to the diversity of the bacterial communities found on the plants inside the warm temperate house (*P=0.02*), but showed no significant difference to the diversity of phyllosphere bacterial communities of the nursery (*P=0.07*) and succulent (*P=1.0*) houses (Table S1). Rarefaction curves of phyllosphere bacterial communities for each plant sample are shown in Fig. S5B. The curves also exhibited low slopes that did not reach saturation. Consequently, Chao1 showed putative OTUs ranging from 344.7-1,271.7 and a coverage of only 23.7 % - 33.3 % of Chao1 (Table 2). On the other hand, computation of Shannon index of diversity (H') per plant revealed a wider range of values, where *Chlorophytum comosum* showed highest at 7.2 and *Epipremnum aureum* showed lowest at 2.5. However, statistical analysis using Two-sample T-Test showed no significant differences in diversity per sample (Table S2).

Drivers of bacterial community structure

In order to determine the uniqueness of the associations of the phyllosphere bacterial communities in relation to room climate and plant host species, ordination analysis, ANOSIM tests, and BIO-ENV were performed. Principal coordinate analysis (PCoA) using Bray-Curtis dissimilarities showed inconspicuous clustering of the phyllosphere bacterial communities (Fig. 4A and B). The phyllosphere bacterial communities of the nursery and succulent house

were highly scattered and were observed to overlap with the phyllosphere bacterial communities found in the warm temperate and cold temperate houses, while the communities in the tropical house were distinctly different from all the other greenhouses with a slight overlap of microbial composition to phyllosphere bacterial communities of the succulent house. Furthermore, phyllosphere bacterial communities in the warm temperate and cold temperate houses were distinctly closer to one another than to the rest of the houses. Nevertheless, ANOSIM results showed weak correlation between room climate and phyllosphere bacterial community composition (*P=0.001, R=0.25*).

On the other hand, ANOSIM showed a stronger correlation between phyllosphere bacterial communities and plant samples (*P=0.001, R=0.66*). This association was attributed to differences in plant-host leaf morphology. It was observed that *Dracaena fragrans*, *Howea fosteriana*, *Dracaena draco*, and *Beaucarnea recurvata,* having a common ensiform (sword-like) leaf-shape, exhibited bacterial communities with smaller distances to each other than to the rest of the sampled plants (Fig. S6). On the other hand, while the two *Musa* species share a common leaf-shape they have different leaf-sizes and exhibit highly distant clusters of bacterial communities.

BIO-ENV analysis provided further evidence of a stronger correlation between bacterial communities and plant species. In Fig. 5, the vectors represent the Spearman rank correlations (ρ_s) between the variables influencing the phyllosphere bacterial community distribution. According to BIO-ENV correlation analysis (Table S3), plant species best explains the dissimilarity of the bacterial community structure (*BEST= 0.9157*), and Fig. 5 showed that the highest degree of influence of the plant species was observed in the phyllosphere of plants from the tropical house, represented by the longest vector. Although humidity and room temperature showed directional influences on the bacterial community distribution BIO-ENV BEST values (Table S3) showed that they have lesser influence compared to the plant species, since their addition to the variable combination generated a lower coefficient (ρ_s) (Clarke & Ainsworth 1993).

Population densities of phyllosphere communities

The highest abundance of culturable bacteria was found on the leaves of *Chlorophytum comosum* where 5.05 x 10^6 and 6.69 x 10^6 CFU cm^{-2} was recorded from the R2A and NA media respectively, while the lowest density was observed on the leaves of *Musa acuminata* with 3.36 x 10^2 (R2A) and 2.94 x 10^2 CFU cm^{-2} (NA) as shown in Table 3. One-way ANOVA results (Table S4) showed that there are significant differences in the bacterial

population densities on the phyllosphere of 14 indoor plants (*F crit=2.5; F=47.9; P<0.001*), and Tukeys HSD test correspondingly showed three groupings of bacterial population densities with significant differences (Table S5). Tukeys HSD grouping showed that CFU observed from *Chlorophytum comosum* was significantly different from the rest of the plant (*P<0.001*) while CFU from *Aechmea eurycorymbus* showed significant difference (*P<0.001*) to the rest of the plant except to *Dracaena marginata (P=0.90)*, and *Musa x paradisiaca (P=0.372)*.

Antagonistic potential of phyllosphere bacterial communities against *B. cinerea*

A total of 1,284 bacterial isolates from the phyllosphere of 14 indoor plants were screened for their antagonistic potential against *B. cinerea* using TCVA. Antagonistic effects observed included inhibition of mycelial growth and spore germination (Fig. S7). Table 3 shows the number of isolates per plant sample and the percentage of bacterial strains that were tested positive for both antagonistic effects. *Beaucarnea recurvata* showed to harbor the highest percentage of antagonistic bacterial strains with 58% of the 96 bacterial isolates exhibiting inhibitory effect on both mycelial growth and spore germination of *B. cinerea*.

This study focused on identifying the 233 isolates that showed optimum antagonistic potential against the model pathogenic fungi. BOX-PCR fingerprinting and analysis further divided these isolates into 49 genotypic groups at 60 % cut-off level and Sanger sequencing identified 24 species out of the 49 genotypic groups (Table 4). Frequently isolated antagonistic bacterial species included: *Bacillus thuringiensis* (10), *Bacillus toyonensis* (6), *Stenotrophomonas rhizophila* (6), *Bacillus cereus* (4), *Kocuria haloterans* (3), and *Kocuria sedeminis* (2). There was a high representation of the genus *Bacillus* with seven different *Bacillus* species identified (*B. thuringiensis, B. licheniformis, B. cerues, B. aures, B. toyonensis, B. anthracis* and *B. subtilis*). Three days after the initial set-up, the VOCs produced by these bacterial species decreased the fungal colony diameter by about 36% – 61% compared to the control. The mean percent inhibitions caused by each bacterial species are also shown in Table 4 and ANOVA results showed no significant differences between the means (Table S6).

Discussion

Recent studies revealed that phyllosphere microbes have the potential to support human health in a built environment (Berg *et al.*, 2014; Mahnert *et al*., 2015). In this study we examined the phyllosphere bacterial community profile of 14 different plants grown under controlled greenhouse conditions. Using this unique experimental design we were able to find out that the plant genotype was the most important driver determining the phyllosphere microbiome structure and function. Moreover, we discovered that a high proportion of phyllosphere-associated bacteria can produce VOCs; many of which were highly active against the fungal plant pathogen *Botrytis cinerea*. This supports the functional role of phyllosphere bacteria to defend against pathogens.

The plant genotype was identified as the most important driver determining the phyllosphere microbiome structure of the 14 indoor plants. All leaves displayed high and individual bacterial diversity, however, at phylum level the composition was very similar. *Proteobacteria* and *Actinobacteria* were often reported on phyllospheres (Whipps *et al*., 2008; Redford *et al*., 2010; Zhang *et al*., 2010). However, contrary to most studies where *Proteobacteria* were reported to be dominant in the phyllosphere (Vorholt, 2012; Izhaki *et al*., 2013), the phylum *Firmicutes* was found to be dominant in the overall sequences of the bacterial community profile of all sampled indoor plants. There are two obvious reasons for the high proportion of *Firmicutes*: i) the controlled greenhouse conditions with mainly higher temperatures than outside and ii) the application of the biopesticide Dipel®, consisting of the *Bacillus thuringiensis* strain. Until now, only short-term effects of *B. thuringiensis* on the microbial population structure were reported (Raymond *et al*., 2010). In contrast, we found the *Bacillus* strain well-established in all investigated leaves. Another remarkable observation was the presence of many sequences assigned to *Deinococcus-Thermus* in all of the plant samples and its high abundance in the phyllosphere of *Aechmea eurycorymbus*, *Nephrolephis cordifolia*, and *Aloe arborescens*. Members of this bacterial phylum are known for their ability to tolerate an array of environmental stresses including resistance to UV radiation (Blasius *et al*., 2008) that can enable them to thrive on the hostile phyllosphere environment. These findings support previous studies suggesting that the presence of this bacterial phylum may be more common in the phyllosphere than previously acknowledged (Redford *et al*., 2010; Shade *et al*., 2013). The bacterial communities inhabiting the leaves of the 14 ornamentals also showed pronounced interspecies variation within and across different greenhouses. These findings support previous reports of interspecies variability in microbial

phyllosphere communities (Whipps *et al*., 2008; Redford *et al.*, 2010) and suggest a stronger influence of the plant species on the structure and composition of their associated bacterial communities. Further investigation of plant species effects on the bacterial community composition revealed community assembly patterns driven by plant leaf morphology. Since the characteristics of microbial communities on leaves was based to a large degree on the phenotypic characteristics of the plant that is ultimately controlled by its genetic background (Whipps *et al*., 2008; Cordier *et al*., 2012; Bodenhausen, 2014), the correlation between leaf morphology and dissimilarity in bacterial assembly implies an effect of the plant genotype on the bacterial community. Thus, in accordance with previous literature, this study demonstrates the direct influence of the plant host on the phyllosphere microbial community composition correlated to the plant genotype (Redford *et al*., 2010; Cordier *et al*., 2012).

Cultivation-dependent techniques revealed that the phyllosphere of 14 indoor plants harbored bacterial isolates that exhibit antagonistic VOCs activity to both the growth and sporulation of *B. cinerea.* A remarkably high frequency of *Bacillus spp*. that are active VOCs producers were isolated from the phyllosphere of all plant samples. *Bacillus spp*. has shown VOCs inhibitory effects against a variety of plant pathogens (Fiddaman & Rosall, 1993; Islam *et al*., 2012). A study by Islam *et al.* (2012) on the antagonistic effect of VOCs produced by *B. subtilis* showed that ethyl acetate crude extract and fraction DG4 (an isomer of acetylbutanediol), induced significant inhibition of mycelial growth of diverse pathogenic fungi. Acetylbutanediol were also isolated from *B. subtilis* GB03 and *B. subtilis* IN937a and was found antagonistic to the pathogenic bacteria *Erwinia carotovora* in *Arabidopsis thaliana* (Ryu *et al*, 2004), suggesting that these compounds have a wide range of antimicrobial effects. Another remarkable observation was the antifungal VOCs production of *Janibacter melonis* and *Deinococcus spp*.; bacterial species that are known for their biodegradation potential. *Janibacter spp*. are capable of degrading aromatic compounds like pentachlorophenol and dioxins making these *Actinobacteria* species important for bioremediation of polluted environments (Hiraishi, 2003; Yamazoe *et al.*, 2004). *Deinococcus spp*., on the other hand, has been reported to be one of the phyllosphere colonizers that degrade phenanthrene; a model polycyclic aromatic hydrocarbon (PAH) that are urban air pollutants (Waight *et al.*, 2007). Production of potential biocontrol compounds has been reported on *Janibacter spp*. The newly discovered tetrahydroquinoline antibiotic, helquinoline, was isolated from *J. limosus and J. melonis* (Asolkar *et al.*, 2004). Tetrahydroquinoline derivatives containing simple or complex substituents are used as antioxidants, corrosion inhibitors, pesticides, and pharmaceutical agents (Asolkar *et al*.,

2004). Alongside this antibiotic production, *J. melonis* was also reported to produce active VOCs that are antagonistic to *Ralstonia solanacearum,* the causative agent of vascular wilt in many crop plants (Achari & Ramesh, 2014). However, reports on the biocontrol potential of *Deinococcus* are still lacking. Thus the results presented here illustrate the biocontrol potential of *Deinococcus* and adds as well to the growing knowledge on the biocontrol capability of *Janibacter*. Aside from the clear defensive effect on their host plants, the volatiles produced by the phyllosphere bacterial colonists are also important for successful establishment of microbial communities. Volatile organic compounds were postulated as important determinants in shaping epiphytic bacterial communities, since bacteria may produce VOCs that can elicit transcriptional changes in their plant-hosts enabling them to control microbial colonizers on their surfaces (Junker & Tholl, 2013). In addition, since adhesion and aggregation on the leaf surface is the dominant lifestyle of phyllosphere microbes (Vorholt, 2012), long-distance mechanisms for antagonism is vital for their survival. Moreover, these organic compounds can serve as ideal signaling molecules in facilitating both short- and long-distance intercellular and organismal interactions (Bitas *et al.*, 2013) due to their ability to move through air spaces (Effmert *et al.*, 2012).

Our results provide insights into the structure and function of phyllosphere bacterial communities of plants grown inside a built environment. It was established that the ambient room climate had little influence on the phyllosphere communities, since plant species exhibited high variation of bacterial community composition within and across different greenhouses. This implies that plants have a stable bacterial diversity composition regardless of the room climatic condition. Plant species having higher influences on the bacterial community composition of their associated microbes can be beneficial in establishing a healthy built environment that is ultimately favorable to human health. Research suggests the possibility of influencing a room microbiome by placing certain plant species indoors (Mahnert *et al*., 2015), which results in an increase of microbial diversity and of beneficial microorganisms (Berg *et al*., 2014). Since epiphytes are capable of degrading compounds that are toxic to plants, humans, and the environment (Bringel & Couée, 2005), they are also helpful to maintain a healthy indoor air quality in built environments. Both plants placed indoors (Orwell *et al*., 2004, Pegas *et al*., 2012) and their associated microbes work cumulatively in improving the air quality by absorbing, degrading, detoxifying, and sequestering air pollutants (Weyens *et al*., 2015). The introduction of plants to built environments has the potential to limit the growth of pathogenic molds and fungi responsible for causing “sick building” syndrome (Strauss, 2009), since the phyllosphere bacterial

community also includes species that produce antifungal volatiles.

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Figures and Tables for:

The plant is crucial: specific composition and function of the phyllosphere microbiome of indoor ornamentals

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Table 1. Species richness estimates (categorized per greenhouse) obtained from 16S rRNA gene amplicons from 14 greenhouse plants.

Greenhouse	Seqs/Sample	Shannon index (H')	Observed species (no. of OTUs)	Chao1 (no. of OTUs)	Coverage (%)
cold temperate		6.6	306.7	1097.1	28.0
nursery		6.1	269.5	918.0	29.4
succulents	714	4.8	209.7	790.9	26.5
warm temperate		5.8	279.4	1065.5	26.2
tropical		4.1	152.0	510.5	29.8

Table 2. Species richness estimates (categorized per plant) obtained from 16S rRNA gene amplicons from 14 greenhouse plants.

Greenhouse	Sample	Origin	Seqs/Sample	Shannon index (H')	Observed species (no. of OTUs)	Chao1 (no. of OTUs)	Coverage (%)
tropical	Bth	*Aechmea eurycorymbus*	714	4.7	178.2	604.8	29.5
	Dth	*Dracaena marginata*		5.5	188.5	592.5	31.8
	Eth	*Epipremnum aureum*		2.5	104.7	344.7	30.4
	Mth	*Musa x paradisiaca*		3.8	136.9	499.9	27.4
warm temperate	Dtm	*Dracaena fragrans*		5.4	276.3	1167.6	23.7
	Htm	*Howea forsteriana*		5.8	279.7	1046.1	26.7
	Mtm	*Malvaviscus penduliflorus*		6.0	282.2	982.7	28.7
nursery	Nnr	*Nephrolepis cordifolia*		6.1	269.5	918.0	29.4
cold temperate	Cch	*Chlorophytum comosum*		7.2	301.4	905.3	33.3
	Dch	*Dracaena draco*		5.8	290.3	1114.3	26.0
	Och	*Olea europaea*		6.8	328.4	1271.7	25.8
succulents	Asa	*Aloe arborescens*		5.2	214.8	801.3	26.8
	Bsa	*Beaucarnea recurvata*		3.6	155.4	536.1	29.0
	Msa	*Musa acuminata*		5.7	258.9	1035.2	25.0

Table 3. Plate counts (CFU cm^{-2}) and percentages of antagonistic bacteria against the plant pathogenic fungi *Botrytis cinerea**.

Greenhouse	Plant species	CFU ± SD		Total isolates tested	Inhibits both growth and sporulation	
		R2A	NA		No. of isolates	%
tropical	*Aechmea eurychorymbus*	1.61E+06 ± 2.95E+04	1.48E+06 ± 1.34E+05	96	2	2
	Dracaena marginata	1.13E+06 ± 1.68E+04	9.01E+05 ± 6.14E+04	96	36	38
	Epipremmum aureum	2.99E+05 ± 1.68E+04	2.04E+05 ± 6.14E+04	96	17	18
	Musa x paradisiaca	8.13E+05 ± 3.92E+05	5.67E+05 ± 2.02E+05	96	1	1
warm temperate	*Dracaena fragrans*	4.53E+04 ± 2.14E+04	8.39E+03 ± 3.75E+03	96	21	22
	Howea forsteriana	2.11E+04 ± 1.89E+04	3.32E+03 ± 1.45E+03	96	35	36
	Malvaviscus penduliflorus	2.41E+04 ± 2.06E+04	3.17E+04 ± 3.00E+04	90	2	2
nursery	*Nephrolepis cordifolia*	1.44E+05 ± 1.04E+05	1.12E+05 ± 1.09E+05	96	23	24
cold temperate	*Chlorophytum comosum*	5.05E+06 ± 2.33E+06	6.69E+06 ± 3.08E+06	96	5	5
	Dracaena draco	4.79E+04 ± 3.45E+04	1.15E+04 ± 5.81E+03	96	6	6
	Olea europaea	1.88E+04 ± 1.61E+04	1.39E+04 ± 1.22E+04	91	22	24
succulents	*Aloe arborescens*	1.15E+05 ± 3.28E+04	1.74E+05 ± 4.68E+04	96	4	4
	Beaucarnea recurvata	9.89E+04 ± 6.18E+03	4.33E+04 ± 8.80E+03	96	56	58
	Musa acuminata	3.36E+02 ± 1.76E+02	2.52E+02 ± 6.24E+01	47	3	6

*Colony forming units (CFU) cm^{-2} shown per growth medium used; Reasoner's 2A (R2A) agar and Nutrient broth II agar (NA) medium.

Table 4. Taxonomic classification and mean growth inhibition percentage against *Botrytis cinerea* of isolated phyllosphere bacterial strains from 14 greenhouse plants.

Greenhouse	Strains	Plant of origin	% inhibition	Inhibition score	Species	Similarity (%)	Accession
tropical	Bth1N11	*Aechmea eurycorymbus*	52.79	++	*Deinococcus grandis* DSM 3963	99	NR_026399.1
	Dth4N3	*Dracaena marginata*	52.14	++	*Bacillus thuringiensis* Bt407	100	NR_102506.1
	Dth4R1		49.93	++	*Bacillus thuringiensis* Bt407	100	NR_102506.1
	Dth3R4		46.99	++	*Bacillus thuringiensis* Bt407	100	NR_102506.1
	Dth3R5		52.79	++	*Bacillus thuringiensis* Bt407	100	NR_102506.1
	Dth1R12		38.86	++	*Staphylococcus saprophyticus* ATCC 15305	99	NR_074999.1
	Dth3R1		49.19	++	*Bacillus thuringiensis* Bt407	100	NR_102506.1
	Dth3N9		41.41	++	*Sphingobium yanoikuyae* NBRC 15102	99	NR_113730.1
	Dth4N9		48.07	++	*Kocuria haloterans* YIM 90716	99	NR_044025.1
	Dth1R9		40.01	++	*Kocuria haloterans YIM 90716*	99	NR_044025.1
	Dth2R10		61.17	+++	*Sphingomonas dokdonensis* DS-4	99	NR_043612.1
	Dth4N5		36.96	++	*Brachybacterium conglomeratum* J1015	99	NR_104689.1
warm temperate	Dtm2N2	*Dracaena fragrans*	61.31	+++	*Bacillus licheniformis* DSM 13	99	NR_118996.1
	Dtm2R12		51.47	++	*Bacillus toyonensis*BCT-7 112	100	NR_121761.1
	Dtm3R12		53.13	++	*Kocuria haloterans* YIM 90716	99	NR_044025.1
	Dtm3N4		50.99	++	*Bacillus cereus*ATCC 14579	99	NR_074540.1
warm temperate	Htm3N10	*Howea forsteriana*	56.18	++	*Bacillus aureus* 24K	100	NR_118439.1
	Htm2N10		36.76	++	*Bacillus toyonensis*BCT-7 112	99	NR_121761.1
	Htm4R1		53.19	++	*Bacillus toyonensis*BCT-7 112	99	NR_121761.1
	Mtm3R3	*Malvaviscus penduliflorus*	49.90	++	*Bacillus cereus*ATCC 14579	99	NR_074540.1
nursery	Nnr2N2	*Nephrolepis cordifolia*	44.56	++	*Micrococcus flavus*LW4	99	NR_043881.1
	Nnr3R10		54.85	++	*Bacillus toyonensis*BCT-7 112	99	NR_121761.1
	Nnr4R12		50.69	++	*Bacillus thuringiensis* Bt407	100	NR_102506.1
	Nnr2R7		40.50	++	*Bacillus anthrasis*strain Ames	99	NR_074453.1
	Nnr3N9		39.41	++	*Kocuria sediminis* FCS-11	99	NR_118222.1
	Nnr2R11		49.17	++	*Bacillus thuringiensis* Bt407	100	NR_102506.1
	Nnr4N11		42.35	++	*Pseudomonas aeruginosa* SNP0614	99	NR_118644.1
	Nnr3N6		42.89	++	*Deinococcus xibeiensis*	99	NR_116670.1
	Nnr1R4		44.64	++	*Sphingobium xenophagum* BN6	99	NR_026304.1
	Nnr3R3		43.73	++	*Janibacter melonis* CM2104	99	NR_025805

[a] *Inhibition score (+) indicates 10-30%; (++) 31-60% ; (+++) 61-80%; (++++) 81-100% growth inhibition effect on Botrytis cinerea*

Table 4. Taxonomic classification and mean growth inhibition percentage against *Botrytis cinerea* of isolated phyllosphere bacterial strains from 14 greenhouse plants.

Greenhouse	Strains	Plant of origin	% inhibition	Inhibition score	Species	Similarity (%)	Accession
cold temperate	Cch2R2	*Chlorophytum comosum*	38.21	++	*Pantoea vagans* C9-1	99	NR_102966.1
	Cch2R9		58.25	++	*Stenotrophomonas rhizophila* e-p10	99	NR_121739.1
	Cch2N9		55.84	++	*Stenotrophomonas rhizophila* e-p10	100	NR_121739.1
	Cch4R6		56.90	++	*Bacillus cereus*ATCC 14579	99	NR_074540.1
	Cch1N3		48.34	++	*Stenotrophomonas rhizophila* e-p10	99	NR_121739.1
	Dch3R8	*Dracaena draco*	45.88	++	*Bacillus thuringiensis* Bt407	100	NR_102506.1
	Dch4R3		53.97	++	*Stenotrophomonas rhizophila* e-p10	98	NR_121739.1
	Och1N4	*Olea europaea*	49.68	++	*Bacillus subtilis* SBMP4	100	NR_118383.1
	Och2R2		42.33	++	*Bacillus thuringiensis* Bt407	99	NR_102506.1
	Och4N12		61.15	+++	*Bacillus thuringiensis* Bt407	100	NR_102506.1
	Och2N7		51.15	++	*Erwinia aphidicola* X001	99	NR_104724.1
	Och4N8		43.41	++	*Bacillus cereus*ATCC 14579	99	NR_074540.1
	Och4R5		51.53	++	*Stenotrophomonas rhizophila* e-p10	100	NR_121739.1
succulents	Asa3N6	*Aloe arborescens*	45.16	++	*Kocuria turfanensis* HO-9042	99	NR_043899.1
	Asa1N1		45.11	++	*Kocuria sediminis* FCS-11	99	NR_118222.1
	Bsa3N9	*Beaucarnea recurvata*	52.81	++	*Bacillus toyonensis*BCT-7 112	100	NR_121761.1
	Bsa3N2		56.29	++	*Bacillus thuringiensis* Bt407	100	NR_102506.1
	Bsa3R7		56.29	++	*Bacillus toyonensis*BCT-7 112	99	NR_121761.1
	Msa2R2	*Musa acuminata*	56.27	++	*Microbacterium oleivorans* BAS69	99	NR_042262.1

[a]*Inhibition score (+) indicates 10-30%; (++) 31-60% ; (+++) 61-80%; (++++) 81-100% growth inhibition effect on Botrytis cinerea*

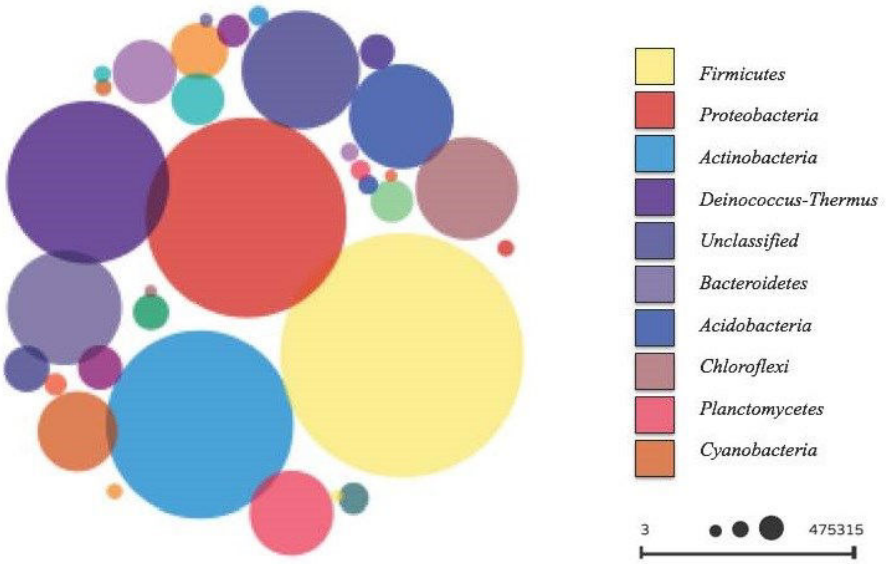

Figure 1. Overview of phyllosphere microbial communities found on 14 different greenhouse plants on phylum level. The size of the spheres indicates the relative abundance of the corresponding phylum; larger spheres indicate higher abundance. Index shows the top 10 most abundant phyla.

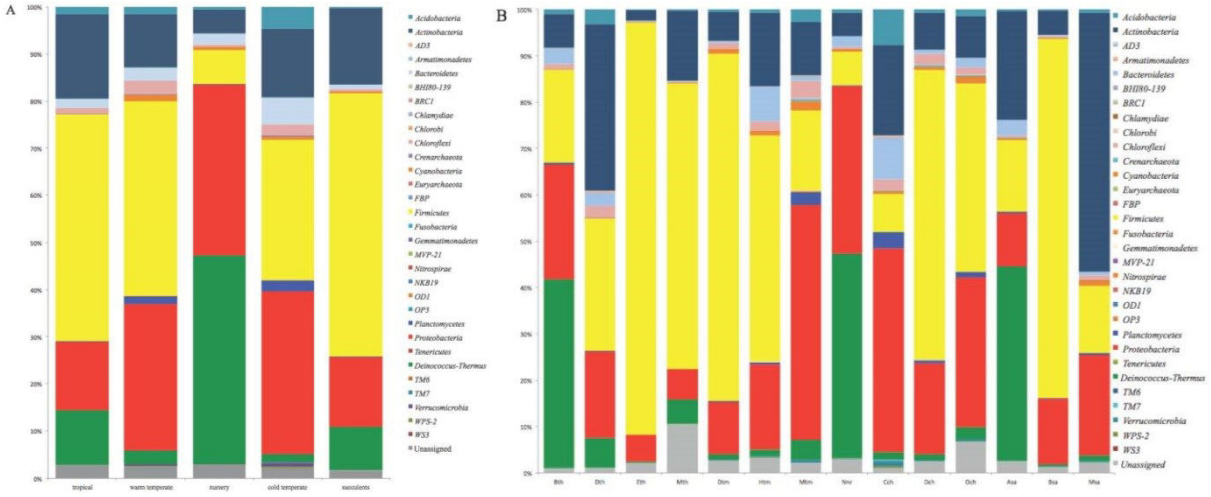

Figure 2. Relative abundance of phyllosphere microbial communities found on 14 different greenhouse plants at phylum level categorized A) per greenhouse and B) per plant species.

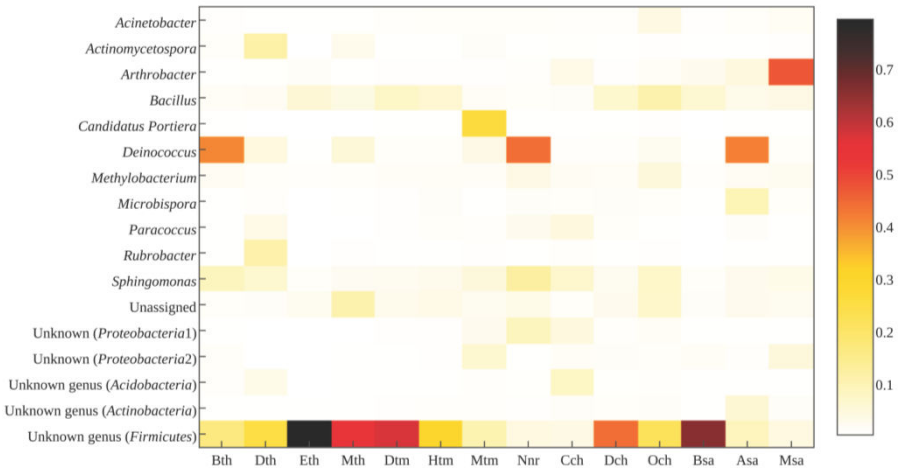

Figure 3. Relative abundance of dominant sequences assigned to genus level (1% cut-off) found on the phyllosphere of 14 different greenhouse plants

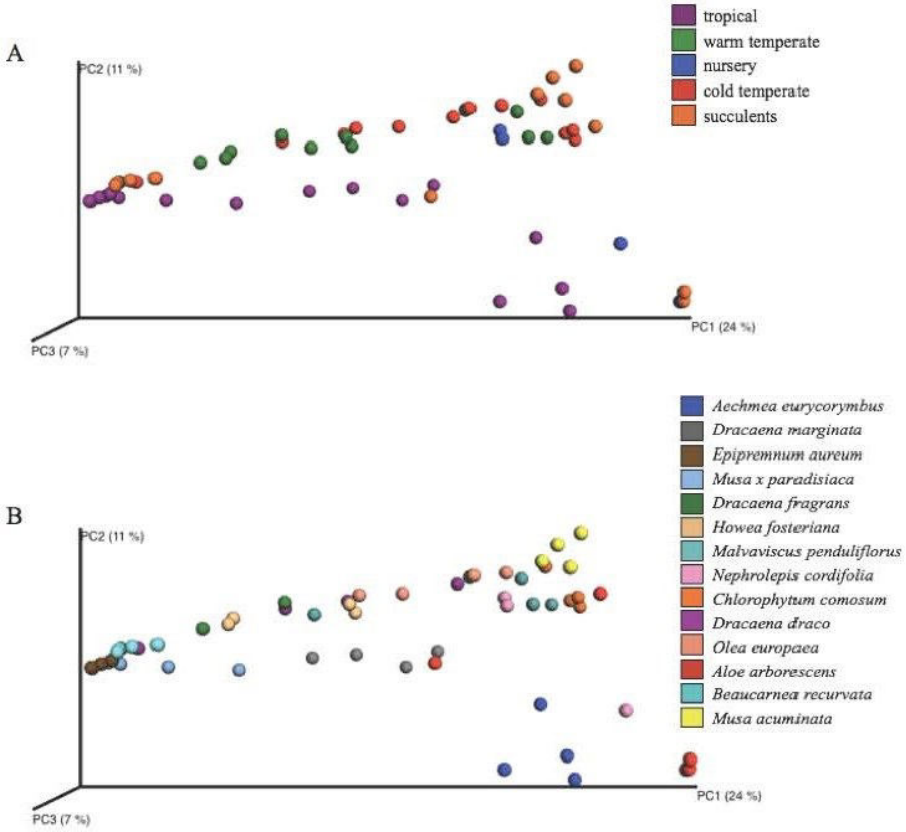

Figure 4. PCoA plots showing the beta-diversity clustering patterns of samples in A) greenhouse areas, and B) plant species, based on Bray-Curtis dissimilarity

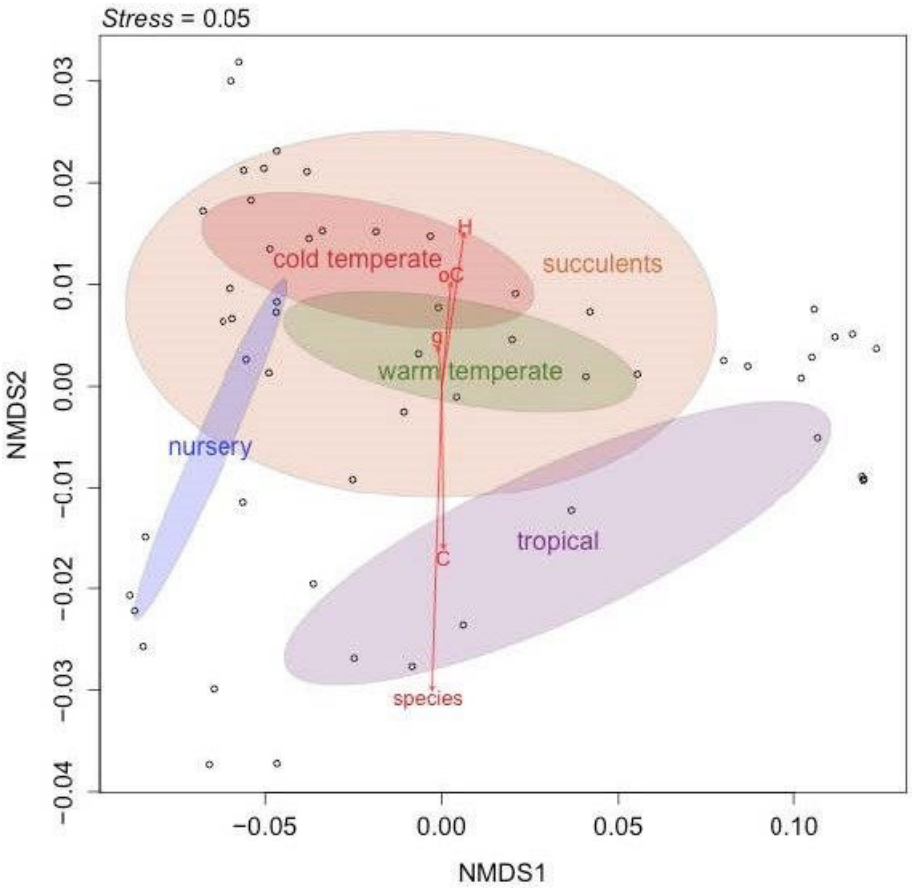

Figure 5. Non-metric multidimensional scaling (Stress = 0.05; 999 permutations) plot based on Bray-Curtis dissimilarities and the influence of biotic and abiotic variables, shown as vectors, on bacterial community composition determined via BIO-ENV analysis based on Euclidean distances. The length of the vectors is proportional to the degree of influence of the corresponding variable. Species = plant species; H = relative humidity inside the room; C = temperature inside the room; oC = temperature outside the room; g = leaf weight.

Supplementary Figures and Tables for:

The plant is crucial: specific composition and function of the phyllosphere microbiome of indoor ornamentals

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Table S1. Statistical analysis using a Two-sample T-test of the Shannon index per greenhouse area.

Group1	Group2	Group1 mean	Group1 std	Group2 mean	Group2 std	t stat	p-value
cold temperate	tropical	6.605629927	1.013899801	4.114037414	1.160754266	5.714483001	0.01
warm temperate	succulents	5.750714363	1.252498113	4.832221754	1.373029359	1.639129568	1.0
tropical	succulents	4.114037414	1.160754266	4.832221754	1.373029359	-1.442715652	1.0
cold temperate	warm temperate	6.605629927	1.013899801	5.750714363	1.252498113	1.759563604	0.97
nursery	warm temperate	6.10252267	1.199731172	5.750714363	1.252498113	0.459852192	1.0
cold temperate	succulents	6.605629927	1.013899801	4.832221754	1.373029359	3.446035259	0.03
nursery	tropical	6.10252267	1.199731172	4.114037414	1.160754266	2.887571852	0.07
nursery	succulents	6.10252267	1.199731172	4.832221754	1.373029359	1.545345381	1.0
cold temperate	nursery	6.605629927	1.013899801	6.10252267	1.199731172	0.766524316	1.0
tropical	warm temperate	4.114037414	1.160754266	5.750714363	1.252498113	-3.438934953	0.02

Table S2. Statistical analysis using a Two-sample T-test of the Shannon index per plant sample.

Group1	Group2	Group1 mean	Group1 std	Group2 mean	Group2 std	t stat	p-value
Nnr	Dch	nan	nan	6.143	1.335	None	None
Nnr	Bsa	nan	nan	3.729	0.325	None	None
Msa	Bth	5.892	1.340	4.819	0.466	1.310	1
Och	Mth	7.070	0.420	3.872	0.437	9.130	1
Mtm	Bsa	6.275	1.636	3.729	0.325	2.645	1
Htm	Bsa	6.015	0.610	3.729	0.325	5.729	1
Dth	Mth	5.634	0.210	3.872	0.437	6.292	1
Dth	Nnr	5.634	0.210	nan	nan	None	None
Bth	Dtm	4.819	0.466	5.660	1.402	-0.986	1
Msa	Dtm	5.892	1.340	5.660	1.402	0.207	1
Mtm	Asa	6.275	1.636	5.377	1.280	0.750	1
Bsa	Dch	3.729	0.325	6.143	1.335	-3.045	1
Eth	Dth	2.618	0.141	5.634	0.210	-20.666	1
Nnr	Bth	nan	nan	4.819	0.466	None	None
Htm	Mth	6.015	0.610	3.872	0.437	4.942	1
Msa	Cch	5.892	1.340	7.452	0.402	-1.931	1
Htm	Asa	6.015	0.610	5.377	1.280	0.779	1
Msa	Dth	5.892	1.340	5.634	0.210	0.328	1
Mtm	Mth	6.275	1.636	3.872	0.437	2.458	1
Mth	Dtm	3.872	0.437	5.660	1.402	-2.109	1
Msa	Och	5.892	1.340	7.070	0.420	-1.453	1
Dth	Cch	5.634	0.210	7.452	0.402	-6.946	1
Mtm	Htm	6.275	1.636	6.015	0.610	0.259	1
Eth	Htm	2.618	0.141	6.015	0.610	-9.393	1
Msa	Mtm	5.892	1.340	6.275	1.636	-0.314	1
Htm	Och	6.015	0.610	7.070	0.420	-2.466	1
Och	Bsa	7.070	0.420	3.729	0.325	10.899	1
Eth	Mth	2.618	0.141	3.872	0.437	-4.727	1
Dch	Dtm	6.143	1.335	5.660	1.402	0.433	1
Htm	Nnr	6.015	0.610	nan	nan	None	None
Eth	Nnr	2.618	0.141	nan	nan	None	None
Nnr	Asa	nan	nan	5.377	1.280	None	None
Dth	Bth	5.634	0.210	4.819	0.466	2.765	1
Mtm	Bth	6.275	1.636	4.819	0.466	1.483	1
Htm	Dch	6.015	0.610	6.143	1.335	-0.152	1
Htm	Cch	6.015	0.610	7.452	0.402	-3.407	1
Bsa	Cch	3.729	0.325	7.452	0.402	-12.487	1
Msa	Nnr	5.892	1.340	nan	nan	None	None
Och	Dtm	7.070	0.420	5.660	1.402	1.669	1
Msa	Mth	5.892	1.340	3.872	0.437	2.481	1
Bth	Dch	4.819	0.466	6.143	1.335	-1.623	1
Bsa	Asa	3.729	0.325	5.377	1.280	-2.162	1
Bth	Cch	4.819	0.466	7.452	0.402	-7.413	1
Eth	Dtm	2.618	0.141	5.660	1.402	-3.740	1
Dth	Bsa	5.634	0.210	3.729	0.325	8.545	1
Mtm	Cch	6.275	1.636	7.452	0.402	-1.209	1
Nnr	Mth	nan	nan	3.872	0.437	None	None

Table S2. Cont.

Group1	Group2	Group1 mean	Group1 std	Group2 mean	Group2 std	t stat	p-value
Asa	Dtm	5.377	1.280	5.660	1.402	-0.258	1
Mtm	Dch	6.275	1.636	6.143	1.335	0.108	1
Eth	Bsa	2.618	0.141	3.729	0.325	-5.437	1
Dth	Dch	5.634	0.210	6.143	1.335	-0.652	1
Eth	Cch	2.618	0.141	7.452	0.402	-19.660	1
Bth	Asa	4.819	0.466	5.377	1.280	-0.710	1
Mth	Cch	3.872	0.437	7.452	0.402	-10.438	0.702
Bsa	Mth	3.729	0.325	3.872	0.437	-0.456	1
Msa	Asa	5.892	1.340	5.377	1.280	0.481	1
Mth	Dch	3.872	0.437	6.143	1.335	-2.800	1
Cch	Dtm	7.452	0.402	5.660	1.402	2.128	1
Mtm	Dtm	6.275	1.636	5.660	1.402	0.495	1
Eth	Bth	2.618	0.141	4.819	0.466	-7.831	1
Och	Dch	7.070	0.420	6.143	1.335	1.147	1
Msa	Htm	5.892	1.340	6.015	0.610	-0.145	1
Htm	Dtm	6.015	0.610	5.660	1.402	0.402	1
Msa	Eth	5.892	1.340	2.618	0.141	4.208	1
Nnr	Och	nan	nan	7.070	0.420	None	None
Mtm	Och	6.275	1.636	7.070	0.420	-0.815	1
Asa	Cch	5.377	1.280	7.452	0.402	-2.679	1
Eth	Dch	2.618	0.141	6.143	1.335	-4.550	1
Och	Bth	7.070	0.420	4.819	0.466	6.214	1
Htm	Bth	6.015	0.610	4.819	0.466	2.698	1
Mth	Asa	3.872	0.437	5.377	1.280	-1.927	1
Dth	Asa	5.634	0.210	5.377	1.280	0.344	1
Htm	Dth	6.015	0.610	5.634	0.210	1.021	1
Eth	Och	2.618	0.141	7.070	0.420	-17.391	1
Bth	Mth	4.819	0.466	3.872	0.437	2.565	1
Mtm	Nnr	6.275	1.636	nan	nan	None	None
Och	Cch	7.070	0.420	7.452	0.402	-1.137	1
Nnr	Dtm	nan	nan	5.660	1.402	None	None
Dth	Dtm	5.634	0.210	5.660	1.402	-0.031	1
Bsa	Dtm	3.729	0.325	5.660	1.402	-2.325	1
Eth	Asa	2.618	0.141	5.377	1.280	-3.712	1
Dch	Asa	6.143	1.335	5.377	1.280	0.718	1
Mtm	Dth	6.275	1.636	5.634	0.210	0.673	1
Dch	Cch	6.143	1.335	7.452	0.402	-1.626	1
Bth	Bsa	4.819	0.466	3.729	0.325	3.325	1
Och	Asa	7.070	0.420	5.377	1.280	2.177	1
Msa	Bsa	5.892	1.340	3.729	0.325	2.717	1
Msa	Dch	5.892	1.340	6.143	1.335	-0.230	1
Eth	Mtm	2.618	0.141	6.275	1.636	-3.859	1
Dth	Och	5.634	0.210	7.070	0.420	-5.294	1
Nnr	Cch	nan	nan	7.452	0.402	None	None

Table S3. Combination of variables giving the largest rank correlation between the biotic/abiotic variables and the bacterial community similarity matrices; bold format indicates the BEST value and variable that explains the distribution of the bacterial community in the phyllosphere of the 14 greenhouse plants.

	Best variable combination (ρ_s)	
Variables	Size	Correlation
species	**1**	**0.9157**
species H	2	0.69
species H C	3	0.4841
species H C oC	4	0.3832
species g H C oC	5	0.3157

**species = plant species; H= relative humidity inside the room; C = temperature inside the room; oC = temperature outside the room; g= leaf weight.*

Table S4. ANOVA of phyllosphere bacterial CFU of 14 different greenhouse plants

Source of Variation	SS	df	MS	F	p-value	F crit
Between Groups	6.31717E+13	13	4.85937E+12	47.87393	2.64276E-09	2.50726
Within Groups	1.42105E+12	14	1.01503E+11			
Total	6.45928E+13	27				

Table S5. Tukeys test of phyllosphere bacterial CFU of 14 different greenhouse plants.

	Plant_samples	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	Msa	2	294.00		
	Htm	2	12194.40		
	Och	2	16365.00		
	Dtm	2	26848.20		
	Mtm	2	27885.00		
	Dch	2	29691.00		
	Bsa	2	71088.60		
	Nnr	2	127839.00		
	Asa	2	144513.00		
	Eth	2	251460.00		
	Mth	2	689760.00	689760.00	
	Dth	2	1016640.00	1016640.00	
	Bth	2		1545480.00	
	Cch	2			5872416.00
	Sig.		0.183	0.372	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 2.0

Table S6. ANOVA of the percent inhibition of antagonistic bacterial strains against the growth of *Botrytis cinerea*

Source of Variation	SS	df	MS	F	p-level	F crit
Between Groups	5.47743	34	0.1611	0.73771	0.84357	1.54108
Within Groups	22.93	105	0.21838			
Total	28.40743	139				

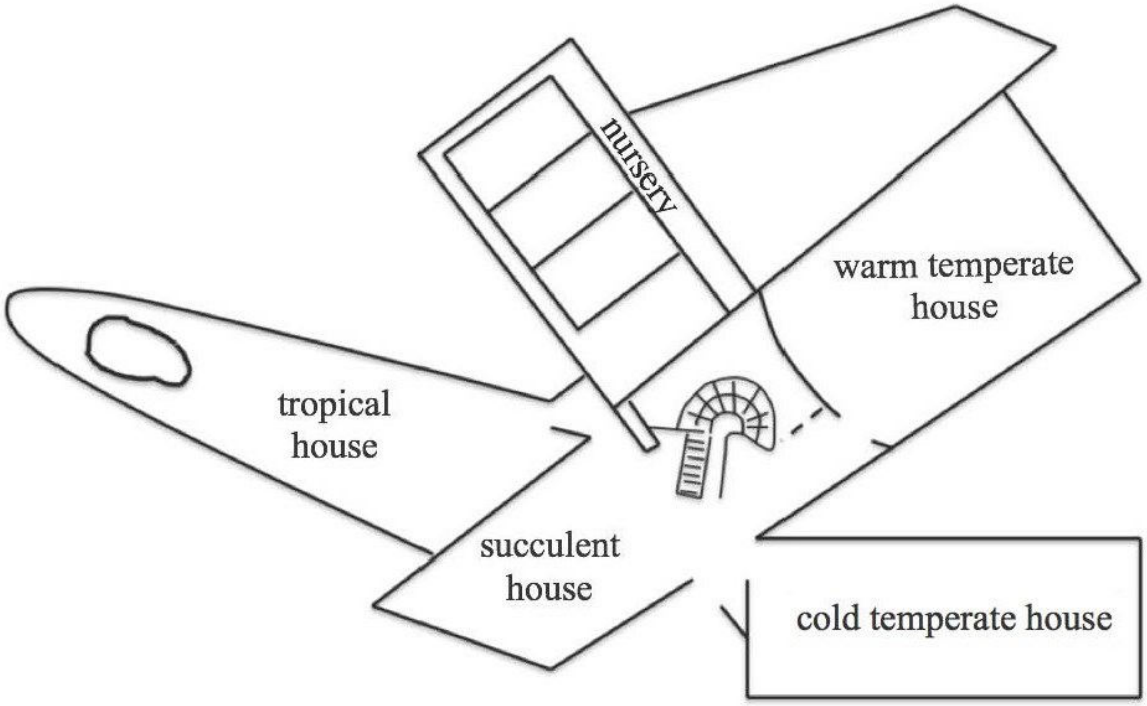

Figure S1. Diagram of the Botanical Garden of Graz greenhouse complex

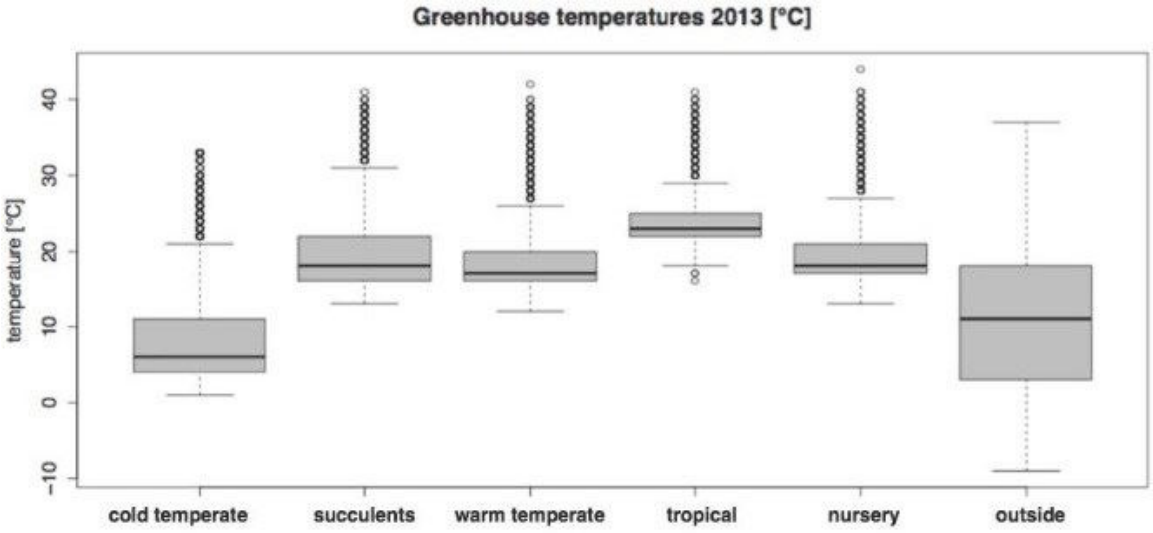

Figure S2. Mean temperature inside each area of the greenhouse complex for the year 2013

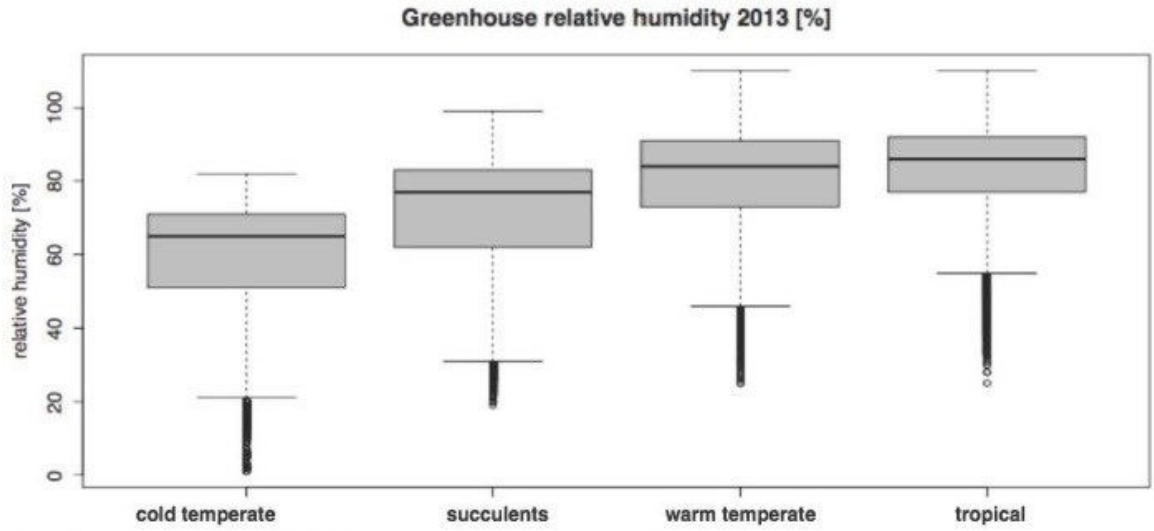

Figure S3. Mean relative humidity inside each area of the greenhouse complex for the year 2013

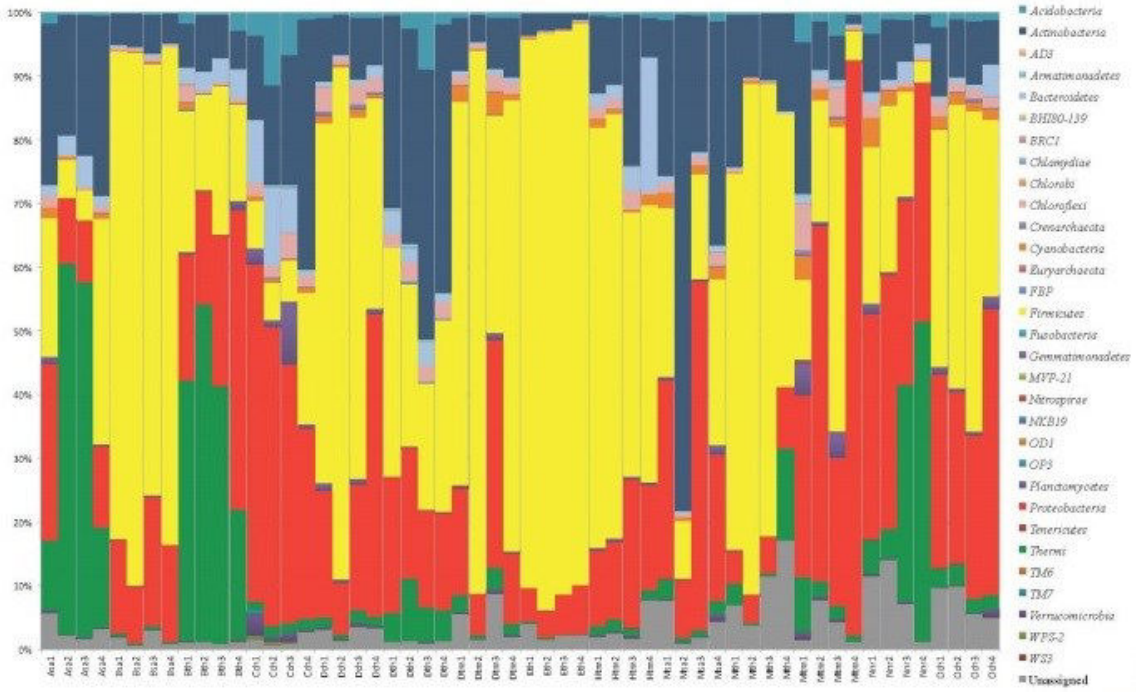

Figure S4. Relative abundance of phyllosphere bacterial community composition from four different replicates of each 14 different greenhouse plants.

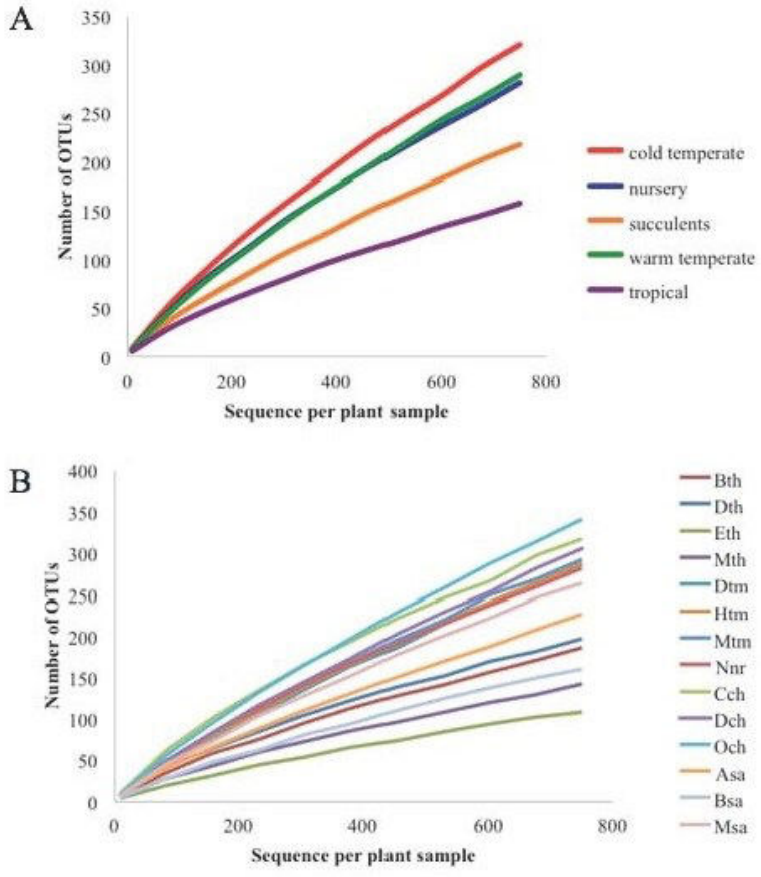

Figure S5. Rarefaction results of the diversity of phyllosphere bacterial communities of 14 greenhouse plants A) per greenhouse and B) per plant species.

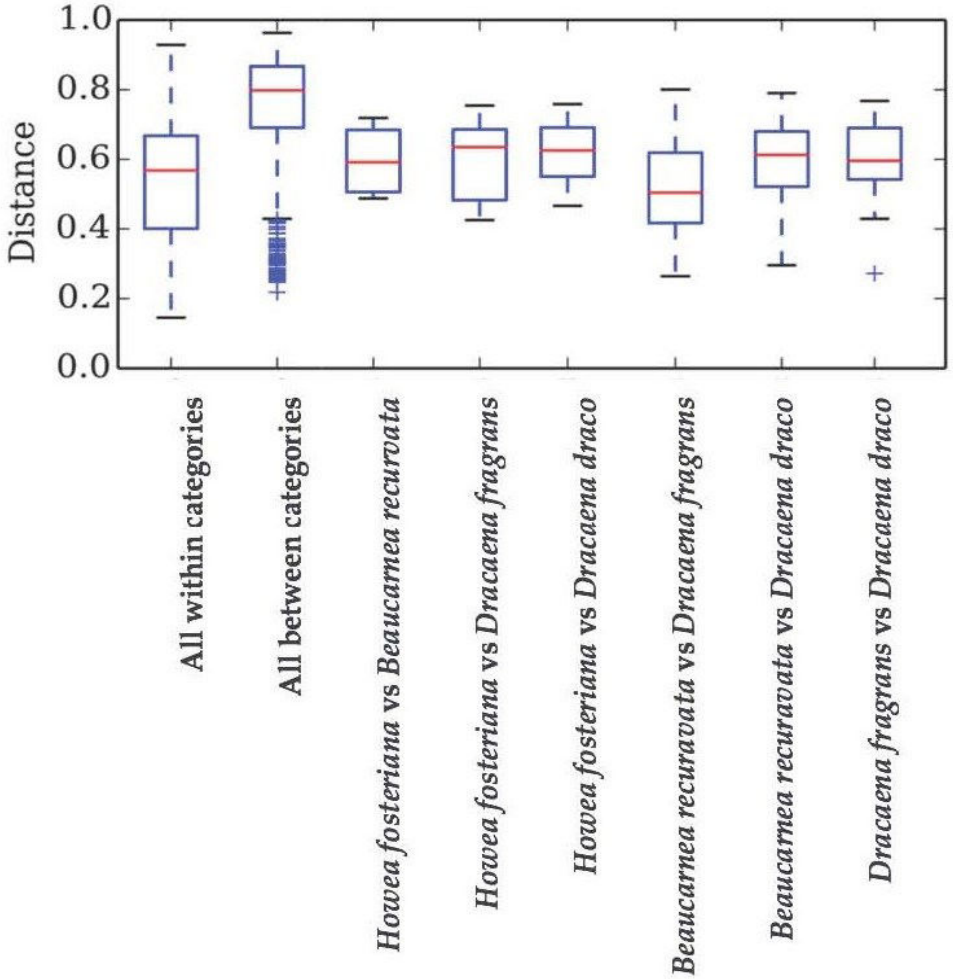

Figure S6. Distances of phyllosphere bacterial communities between plant samples with common leaf shape.

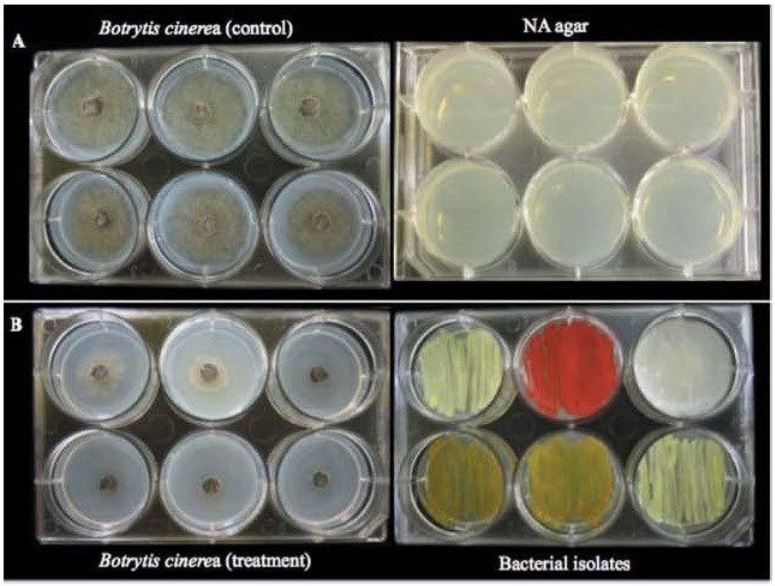

Figure S7. Two-clamp VOCs assay of bacterial isolates from 14 greenhouse plants showing antifungal volatile activity against *Botrytis cinerea*. (A) *B. cinerea* plugs showing mycelial growth and spore germination (left), paired with an empty NA plate (right). (B) Volatile organic compounds produced by bacteria (right) affected mycelial growth and germination of spores (left), compared to the control (above left).

Plant-host taxonomy and genotype as drivers of fungal community structure and the antagonistic potential of fungi on the leaves of greenhouse plants

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(In preparation)

Abstract

Exploration of the phyllosphere community revealed its many beneficial effects on plants and in built environments. Whereas most reports focus on bacterial communities on the phyllosphere a number of literatures showed that the foliar surface also hosts fungal colonists. Thus far, there is limited understanding of the fungal community structure in the phyllosphere of plants, and even less in plants found inside a built environment and the relationship of the fungal community structure to plant-host and the ambient room microclimate. This study investigated 14 common houseplants of diverse plant species and morphology grown in different controlled microclimate in the greenhouse of the Botanical Garden of Graz using cultivation dependent analysis and internal transcribed spacer (ITS) region amplicon sequencing on Illumina MiSeq. Furthermore, the antagonistic potential of fungal isolates was assessed in a Two-clamps volatile organic compounds (VOCs) assay against the model pathogenic fungi *Botrytis cinerea*.

Individual plant species showed high fungal abundance and diversity. The highest population density of culturable fungi was found on the leaves of *Musa paradisiaca* where 1.07 x 106 and 7.32 x 105 CFU cm-2 were recorded from Synthetic Nutrient Agar and Sabouraud growth media, respectively. Abundant fungi identified belonged to order *Capniodiales* from phylum *Ascomycota*, and order *Wallemialles* and *Tremellales* from phylum *Basidiomycota.* Non-metric multidimensional scaling and BIO-ENV analysis also showed correlation of fungal community highly inclined to plant species, where the variability of the community composition is correlated to plant genotype. Fungi isolated from the phyllosphere also exhibited VOCs-based antifungal activity; inhibiting *B. cinerea* mycelial growth by 32.89% - 72.23%. Frequently isolated active VOCs produces were mainly *Penicillum* species along with *Cladosporium*, and *Cryptococcus* species.

This study indicates that plants grown indoors support distinctive fungal communities that features antagonistic potential, and harbors a stable phyllosphere microbial diversity regardless of microclimate and abiotic conditions of a room. Hence, these plants maintain their microbiome independently from their surroundings that could have beneficial effects on microbial diversity and our health inside buildings in general.

Keywords: Microclimate, correlation to genotypic distance of plants, indoor plants, built environments

Introduction

The aboveground parts of a living plant provide a habitat for microorganisms known as the phyllosphere. This habitat is dominated by the leaves with an estimated global leaf area of 109 km2 making it one of the largest microbial habitats on earth (Woodward and Lomas, 2004). For this reason, most studies about the phyllosphere focused mainly on plant leaves. Despite being a hostile environment with rapidly fluctuating solar radiation, temperature, humidity, and nutrient limitation, the phyllosphere supports diverse and complex microbial communities including many genera of bacteria, archaea, filamentous fungi, and yeasts (Lindow and Brandl, 2003; Whipps et al, 2008; Vorholt, JA, 2012). It has been established that the microbial community structure of the phyllosphere is affected by both environmental and biotic factors (Whipps et al, 2008; Vorholt, 2012; Rastogi et al, 2013). Plant-environment interaction controls the prevailing conditions in the plant phyllosphere, determining the establishment of microorganisms on the leaf surface, and influencing microbial colonization (O'Brien and Lindow, 1989; Whipps et al, 2008; Vorholt 2012). For example, biogeography of organisms on the plant surface distinguishes habitat zones that are differentiated based on physical environment (temperature and moisture) and availability of food (photosynthates) (Andrews and Harris, 2000).

Most literatures describing phyllosphere microbial community focus mainly on bacterial colonizers of the leaf, however the leaf surface also supports diverse fungal community (see for example: Santamariá and Bayman, 2005; Kharwar et al, 2010). Compared to their bacterial counterparts, little is known about fungal function on leaf surfaces and fungal community structure. Although reports on the impact of phyllosphere fungal communities on the fitness of their host plant, and their contribution to key processes in the sustainable function of plant ecosystem including nutrient cycling and water transport has been established (Herre et al, 2007; Sunshine et al, 2009; Vujanovic et al 2012).

Hitherto, no general conclusion has been formulated regarding major drivers of fungal phyllosphere composition since not one unifying factor was identified affecting overall fungal phyllosphere assembly. Studies on the fungal communities in the phyllosphere of oak trees (*Quercus macrocarpa*) under rural or urban management practices showed that landuse was the major driver in determining the fungal community composition, diversity, and richness on oak tree leaves (Jumpponen and Jones, 2009). On the other hand, a study on spatial variation in fungal communities of European beech trees (*Fagus sylvatica*) identified host genetics as a

determinant of fungal community assembly on the foliage of beech leaves (Cordier et al, 2012), whereas pyrosequencing analysis of balsam poplar (*Populus balsamifera* L.) phyllosphere recognized plant genotype as the driver of fungal foliar community composition (Bálint et al, 2013).

Aside from investigations of phyllosphere fungal community, there is also increasing literatures reporting fungal volatile organic compounds (VOCs) and their ecological roles. These compounds serve as ideal signaling molecules in facilitating both short and long-distance intercellular and organismal interactions (Bitas et al, 2013) because of their ability to move through air spaces as well as liquids (Effmert et al. 2012). According to the review by Hung et al (2015), fungal VOCs are useful indirect indicators of fungal growth in agriculture, in monitoring spoilage, for chemotaxonomy purposes, for use in biofilters and for biodiesel, plant and animal disease detection, for "mycofumigation", and with respect to plant health. Despite the growing interest on fungal community associated to the phyllosphere, there is limited information about the diversity of phyllosphere fungal communities on the surface of the leaves of plants grown inside a built environment. Most literatures described drivers of community structure, and adaption leading to establishment of these microorganisms on the phyllosphere of field-grown plants or forest stand tree species (see for example: Pereira et al, 2002; Rastogi et al, 2013; Coleman-Derr, 2015).

This study analyzed the fungal community composition on the leaf surface of 14 greenhouse plants from different rooms with different controlled microclimates. It aimed to show the effect of (1) room microclimate in a built system, and (2) plant host on the phyllosphere fungal community assembly. The study also focused on characterization of a long-distance mechanism for antagonism. Using TCVA, the antagonistic potential of culturable fungal strains isolated from the 14 greenhouse plants were tested to determine their antagonistic potential against a model pathogenic fungi *Botrytis cinerea*.

Materials and Methods

Site description and plant maintenance inside the greenhouse

Samples were collected from a greenhouse at The Botanical Garden of Graz. The botanical garden can be found in Graz, Austria at 47°04'55" N, 15°27'28" E, with an elevation of 378 m above sea level. The greenhouse complex has four different rooms simulating different terrestrial climatic conditions and a nursery room where all the young plants and seedlings can be found (Figure S1).

Plants-care measures for the greenhouse plants include watering, and fertilizer and microbial pesticide application. Watering of plants in different rooms vary in regularity and is dependent on the different seasons. Plants in the Cold room, for example, are watered only in the morning during winter, while those found in the Tropical room are watered more frequently. The Botanical Garden has a cistern that catches rainwater and this serves as reservoir for watering of the plants.

Two types of fertilizer are used to help maintain healthy plants: 1) is a NPK liquid fertilizer for foliar application (Wuxal® Top N), and 2) is a water-soluble Phosphate and Potash nutrient (Hakaphos® Rot 8+12+24+(4)) applied in the soil. Application of these fertilizers also varies depending on the state of plant health.

The biological pesticide DiPel® is also used to protect the leaves of greenhouse plants from *Lepidoptera* larvae (caterpillar) that forages on them. This pesticide contains the naturally occurring bacteria *Bacillus thuringiensis kurstaki* known for its toxicity on caterpillars. DiPel® application is done when there is an apparent infestation of Lepidopteran larvae, and is applied by spraying the solution on the leaves of affected plants. Along with foliar fertilizer and microbial pesticide, a non-ionic surfactant (Break Thru® S240) is also applied to safeguard the effectiveness of the treatments. Both measures were done in all greenhouse rooms except the Nursery room.

Sample collection

Leaves of 14 species of indoor plants were collected using ethanol-washed disposable gloves and sterile instruments. They were separated from the rest of the plant by cutting from the base of

the petiole avoiding any possible contact with the leaf blade. Immediately after collection samples were placed inside 25 x 32 cm freezer bags (ARO freezer bags, Düsseldorf, Germany) and stored in a portable cooler with ice packs (GIO'STYLE Colombo Smart Plastics, Italy). All samples were immediately transported back to the laboratory at the Institute of Environmental Biotechnology, Graz University of Technology (TU Graz), Graz, Austria for microbial isolation and DNA extraction.

Fungal isolation

To wash the microbial cells off the leaves, 720 cm2 of leaf sample was placed inside a freezer bag (doubled as precaution from wear and tear) containing 50 ml 0.85% NaCl solution with Tween 80 and was subjected to a series of washing and vortexing. Washing was done by subjecting the leaf through bag-mixer treatment (BagMixer Interscience, St. Nom, France) for 3 minutes. This step is immediately followed by vortexing, using a Transsonic Digital T910 DH sonicator (Elma™, Singen, Germany), for 3 min at 60 Hz. Right after the first sonication step, bag mixer treatment for 1 min, sonication for 3 min at 60 Hz, and a final bag mixing for 1 min, follows consecutively, to complete the series.

The resulting microbial solution was then transferred to a 50 ml Sarstedt tube. For culture-dependent experiments, 100 µl out of the 50 ml solution was serially diluted ten-folds and plated on both Synthetic nutrient agar (SNA; 0.2 g Glucose, 0.2 g Sucrose, 1 g KH2PO4, 1 g KNO3, 0.5 g KCl, 0.5 g MgSO47H2O, and 22 g agar per liter distilled water; adjusted to 5.5 pH with 1 M NaOH) and Sabouraud agar media (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) in duplicates. Then, the remaining microbial solution was centrifuged (using Sorvall RC-5B Refrigerated Superspeed Centrifuge; DuPont Instruments™, USA) at 6,169 g for 20 min to pellet cells. The moist pellets were then transferred to a 2.0 ml sterile Eppendorf tubes and were further centrifuged at 18,000 g for 20 minutes (HERMLE Labor Technik, Germany). Pellets obtained from this final process were then frozen at −70°C until it was used for DNA extraction.

Determination of fungal colony forming units (CFU)

Agar plates, where serially diluted 100 µl of microbial solution was plated, were incubated at room temperature for 5 days. Colony count was done on the fifth day and final counts were expressed as CFU log10 cm^{-2} leaf.

Each colony with distinct phenotype was transferred initially into a tissue culture dish (60 x 15 mm) with Potato dextrose agar (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) medium and were incubated for 5 days to make sure the isolate was not contaminated. Cubes (1 cm^2) of samples from clean isolates were cut and transferred to 2 ml Eppendorf tubes with 1 ml fungi preservation solution (120 ml Glycerin, 40 ml 50% Glucose (stirred overnight), 20 ml Peptone (20%), 20 ml Yeast extract (10%); prepared and autoclaved separately and was numbered according to plant sample genus (except for *Aechmea eurycorymbus* where the common name Bromelia was used as a reference), origin, and isolation medium (e.g. Dth1N1: fungal isolate from *Draceana* from the Tropical house grown on NB II medium). All isolates were kept in a refrigerator (-70°C) at the Institute of Environmental Biotechnology, TU Graz, Graz, Austria.

Internal transcribed spacer (ITS) profiling using MiSeq Illumina Sequencing

DNA extraction: Genomic DNA was extracted using FastDNA® SPIN kit for soil (MP Biomedicals, Solon, OH, USA) as directed in the instruction manual with a revised first step, where pellets (from microbial isolation) instead of soil sample was used. A total of 56 DNA samples were extracted; four replicates for each of the 14 plant samples.

ITS gene amplification: PCR amplifications targeting the ITS region were conducted for each of the 56 samples using ITS1F/ITS2rP primers carrying sample-specific tags (Schoch et al, 2012; White et al, 1990; Gardes and Bruns, 1993). Using the thermocycler TC-Plus (TECHNE, Staffordshire OSA, UK), DNA was amplified in triplicate PCR reactions (30 µl each); 0.9 µl MgCl (25 mM), 6 µl Taq & Go, 1.5 µl of 5 µM for each primer, 19.1 µl PCR water, and 1µl of the DNA template (95°C, 5 min; 30 cycles of 95°C, 30 s; 58°C, 35 s; 72°C 40 s; and elongation at 72°C, 10s). Amplicons from three independent reactions were then pooled and purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA).

MiSeq Illumina sequencing: Purified amplicon samples were pooled in equimolar concentrations (520.8 ng DNA) and 50 µg of DNA was sent for Illumina MiSeq sequencing

(Eurofins Genomics, Ebersberg, Germany) with chemistry version 2 (2 x 250bp). Quality controls, indexing of PCR products, sequencing, library preparations and initial filtering of raw reads were conducted by Eurofins Genomics, Ebersberg, Germany.

Bioinformatics and Statistics

After initial quality control raw reads were filtered, stiched and sorted according to respective barcodes. Raw reads are deposited as the project XXX in the European Nucleotide Archive (www.ebi.ac.uk). Stiched reads were processed according to the fungal ITS analysis tutorial in QIIME 1.9.0 (Caporaso et al., 2010; Mahnert et al., 2015). After extracting barcodes per respective lengths, reads were demultiplexed, trimmed and filtered. Chimeric sequences were identified with usearch (Edgar, 2010) providing the QIIME formatted UNITE representative sequences (sh_refs_qiime_ver7_dynamic_01.08.2015) as a reference. Subsequently, all chimeric sequences were removed from the data set. OTUs (operational taxonomic units) were picked in several steps against the UNITE reference given above using the blast algorithm and all remaining sequences were clustered de novo. The resulting OTU table was filtered for single and doubletons before it was rarefied to a depth of 10,419 sequences and served with all metadata as input for following alpha and beta diversity analysis and statistics.

Adonis, ANOSIM (analysis of similarities), ANOVA (analysis of variances), MRPP (multi response permutation procedure), BioEnv, and mantel tests were calculated in QIIME and R (vegan package) with 999 permutations (R Core Team, 2014; Oksanen, 2014; Fierer et al., 2010).

Screening of fungal strains for antagonism to *B. cinerea*

Two-clamp volatile organic compounds assay (TCVA) was done to analyze the antagonistic property of the volatiles produced by the fungal samples. *Botrytis cinerea*, maintained on a Potato Dextrose Agar (PDA), from the Institute of Environmental Biotechnology at TU Graz was used as the model pathogen for this study. TCVA was employed since it was reported a good method in screening for strain-specific antagonistic effect (Cernava et al, 2015). Fungal inoculum was prepared by growing the fungus for 6 days on fresh PDA medium. After this period, *B. cinerea* isolate was observed to have well-developed hyphae and is already sporulating.

TCVA with B. cinerea and fungal isolates from 14 greenhouse plants: A total of 629 fungal isolates were screened for their antagonistic activity against the pathogenic fungi *B. cinerea*. Samples from the stored isolates were transferred onto a new a Petri dish with PDA media and incubated for 6 days; to make sure the isolates were clean. A 5mm sample from plates that showed no contamination were cut and transferred onto a 6-well PDA plate and incubated at room temperature for 3-days. After the incubation period, plates with samples observed positive for growth were clamped together with newly made *B. cinerea* 6-well plates. *B. cinerea*-containing plates were prepared by cutting 5 mm plugs from a 6-day old *B. cinerea* inoculum plate and placing it on the center of each well of a 6-well plate with SNA media.

Setting up of the plate-pair was done based on the set-up suggested by Cernava et al (2015) and was done in quadruplicates. The set-up is incubated at room temperature for 3 days under dark conditions to eliminate any light-induced effect on the experiment (Mares et al, 2004). Inhibition of growth was measured as percent (%) inhibition and was calculated using the following equation:

$$\% \text{ inhibition} = \frac{\text{diameter of fungi (control) - diameter of fungi (with VOCs)}}{\text{diameter of fungi (control)}} \times 100$$

BOX polymerase chain reaction (BOX-PCR) genetic fingerprinting

DNA extraction: BOX-PCR fingerprint analysis of the antagonistic fungal isolates was done to avoid analysis of genetically similar strains. To extract fungal DNA, stored fungal samples were transferred onto different PDA plates for reactivation and were incubated for 3 days at room temperature. After incubation, approximately 3 mm2 of sample positive for growth and clear of contamination was cut for homogenization. After the removal of the adhered agar, each isolate sample was mixed in100 µl of double distilled water with 200 mg glass beads (0.1 - 0.25 mm) in a 2-µl microtube with cap (SARSTEDT, Germany) and was homogenized using MP FastPrep-24 sample preparation system ribolyser (Irvin, Calif., USA) (30 s; 6 ms^{-}). Homogenized samples were then frozen for 30 min at -20°C. Using a heat block (specs) the frozen samples were then heated at 100°C and immediately centrifuged at 16,000 g for 5 min at 4°C (HERMLE Labor Technik, Germany).

Gene amplification, fingerprint generation and evaluation: The PCR reaction mix (25 µl) that was used consist of 1 µl of the extracted DNA, 5 µl of Taq&Go, 2.50 µl of 100 pmol ml- BOX A1R primer (5' CTA CGG CAA GGC GAC GCT GAC G 3'), and 16.50 µl PCR water. Amplification was done using the Tpersonal Combi, Biometra thermocycler (Biometra GmbH, Germany) with an initial denaturation at 95°C for 6 min, followed by 35 cycles of 94°C for 1 min, 53°C for 1 min, and 65°C for 8 min with a final extension at 65°C for 16 min. After amplification, an aliquot of 12 µl PCR product was separated by gel electrophoresis in 1.5 % agarose gel in 0.5 x TBE buffer for 4 h. Then the gel was stained with ethidium bromide and photographed under UV transillumination using GelDoc 2000 (BIORAD, USA). The resulting BOX-PCR fingerprints were evaluated using the GelCompar program (Kortrijk, Belgium) Cluster analysis was done using unweighted pair-group average (UPGMA) algorithm.

Fungal strain identification via SANGER sequencing

DNA extraction: A representative strain from the different BOX clusters produced was used for DNA extraction. Each strain was grown on different PDA plates and incubated for 3 days at room temperature. After incubation, about 1.0 cm^2 of fungal mycel was transferred into a 2 ml microtube with cap filled with small (0.25 -0.5 mm) and medium glass beads (1.5 mm); 200 g each. In each tube, 450 µl extraction buffer (0.2 M tris-HCl (pH 8.5), 0.25 M NaCl, and 1 % (w/v) SDS) was then added and the fungal strains were homogenized using MP FastPrep-24 sample preparation system ribolyser (Irvin, Calif., USA); work done on ice. A volume of 3.0 M sodium acetate (pH 5.2) was added when the mycel appears completely homogenous and each tube was then vortexed, incubated for at least 10 min at -20°C, and centrifuged at 10,000 g for 10 min at 4°C (HERMLE Labor Technik, Germany). The supernatants were then transferred into new 2 µl Eppendorf tubes and one volume of chloroform/isoamylalcohol (24:1) was added in each tube, after which the tubes were inverted for 1 min and centrifuged at 10,000 g for 10 min at 4°C. The resulting aqueous phase for each tube was then transferred into new 2 µl Eppendorf tubes and one volume of phenol was added. Once again the tubes were inverted for 1 min and centrifuged at 10,000 g for 10 min at 4°C. After centrifugation, the aqueous phase was once again transferred to a new 2 µl Eppendorf tube and one volume of isopropanol was added before the tubes were vortexed, incubate for 5 min at room temperature, and centrifuged at 10,000 g for 10 min at 4°C. The resulting supernatant was discarded this time and the pellets were washed with

500 µl of 70% ice-cold ethanol, after which centrifugation followed (10,000 g; 10 min; 4°C). Finally the supernatant was decanted and the pellets were dried under laminar flow, then the DNA was dissolved in in 50 µl TE buffer (SIGMA-ALDRICH CHEMIE GmbH, Steinheim, Germany).

Gene amplification and SANGER sequencing: After DNA extraction, PCR amplification for each strain was performed using 30 µl of PCR reaction mix (0.9 µl MgCl (25 mM), 6 µl Taq & Go, 1.5 µl of ITS1f primer, 1.5 µl of ITS4 primer, 19.1 µl PCR water, and 1µl of the DNA template. Amplification was done with an initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 58°C for 35s, and 72°C for 40 s with a final extension at 72°C for 10 min using a TC-Plus thermocycler (TECHNE, Staffordshire OSA, UK). After amplification, an aliquot of 5 µl PCR product was separated by gel electrophoresis in 0.8 % agarose gel in 1 x TAE buffer for 1 h. Then the gel was stained with ethidium bromide and photographed under UV transillumination using GelDoc 2000 (BIORAD, USA).

Amplicons were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA), then nucleic acid was quantitated using Nanodrop 2000c spectrophotometer (PeQlab, VWR International GmbH, Erlangen, Germany) before the template DNA solution were prepared for SANGER sequencing. A 14 µl DNA solution with 40 ng μl^{-1} concentration and a specific primer (ITS1f) were sent to LGC Genomics (Berlin, Germany) for sequencing.

SANGER sequencing analysis: Sequences were identified into specific fungal species using the BLAST algorithm against the NCBI Targeted Loci Nucleotide BLAST - Internal transcribed spacer region (ITS) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

Population densities of fungal communities on the phyllosphere of 14 greenhouse plants

A high abundance of culturable fungi was observed in the phyllosphere of 14 greenhouse plants, with the highest abundance of CFU cm^{-2} found on the leaves of *Musa paradisiaca* with 1.07 x 10^6 (SNA) and 7.32 x 10^5 (Sabouraud) CFU cm-2 and the lowest on the leaves of *Musa acuminata* with 984 (SNA) and 450 CFU cm^{-2} (Sabouraud) as shown in Table 1. One-way ANOVA results (Table S1) showed that there are significant differences in the fungal population densities on the phyllosphere of 14 greenhouse plants (*F crit=2.5; F=13.8; p-level<0.05*) and Tukey's HSD test also showed 2 groupings of population densities per plant (Table S2) where *Musa paradisiaca* was grouped differently from the rest of the plant (Table 1).

Fungal communities associated with the phyllosphere of 14 greenhouse plants

A total of 6 x 10^6 sequences affiliated to fungi (and few protozoa) were generated for the 14 plant samples of different species. The average sequence per sample was 101,310; ranging from 3 967 to 250,429. An average of 14,220 operational taxonomic units (OTUs) per sample were identified. The majority of the fungal sequences were assigned to Order *Capniodiales* (33.0%) from the phylum *Ascomycota*, and *Wallemialles* (20.14%) and *Tremellales* (16.71%) from the phylum *Basidiomycota* (Figure 1). Members of the *Basidiomycetuos* yeasts belonging to *Sporidiobolales* (6.30%), and *Ascomycetous* fungi from orders *Eurotiales* (5.67%) and *Pleosporales* (4.02%), were also found relatively abundant.

Across different greenhouse rooms diverse fungal community and dominant fungal taxa were observed. Order *Capniodiales*, was most abundant in the phyllosphere of plants from the Temperate (48.95%) and Cold (58.91%) rooms, *Wallemiales* in the Succulent room (49.0%), *Tremellales* in the Tropical room (25.85%), and *Sporidiobolales* in the Nursery room (58.91%) as shown in Figure 2.

Fungal abundance per plant sample also showed remarkable variation in their associated fungi. Figure 3 shows that *Capnodiales* was most abundant in the phyllosphere of *Musa paradisiaca* (72.76%) *Dracaena fragrans* (65.29%), *Howea fosteriana* (60.64%), *Malvaviscus penduliflorus* (20.91%), *Chlorophytum comosum* (55.87%), *Dracaena draco* (64.65%), and *Olea europaea*

(56.22%), while class *Wallemiales* was found most abundant in the phyllosphere of *Epipremnum aureum* (79.65%), *Beaucarnea recurvata* (83.47%), *and Musa acuminata* (58.11%). On the other hand, *Tremellales* was most abundant in the phyllosphere of *Dracaena marginata* (73.17%) and *Aloe arborescens* (78.59%), whereas *Sporidiobolales* in *Aechmea eurycorymbus* (42.45%), and *Nephrolepis cordifolia* (33.45%).

Variability in the taxonomic structure of the phyllosphere community was also observed for the different replicates of each plant sample (Figure 4). In comparison to the interspecies variation in fungal diversity shown in Figure 3, Figure 4 shows there is less intraspecies variation for all plant samples, except for *Nephrolepis cordifolia*; which exhibits younger leaves compared to the rest of the plants.

The relative abundance of dominant genus was also analyzed at 1% cut-off level and shown in Figure 5. Analysis showed that dominant genera include *Cladosporium* (31.0%), *Wallemia* (20.1%), and *Cryptococcus* (16.6%). Consequently, these three genera were found ubiquitous in all the plant samples.

Alpha diversity patterns

Rarefaction analysis together with Chao1 value revealed variation in the phyllosphere fungal communities per greenhouse room and per plant sample.

The rarefaction curve for each greenhouse did not reach saturation (Figure 6) and the number of OTUs observed covered 45.8% - 53.08% of the estimated taxonomic richness (Chao1) as seen in Table 2. The computed Shannon index of diversity (H′) is varied where it was highest in the phyllosphere fungal community in the Nursery room (6.5) and is lowest in the Succulent room (4.2). Statistical analysis using Twosample T-Test showed that H′ of phyllosphere fungal communities in the Nursery room is significantly different from the Cold room but not to the rest of the rooms, while the H′ of the phyllosphere fungal community in the Tropical house is significantly different from the Temperate and Cold rooms, and no significant difference between the H′ of the Cold and Temperate rooms was observed (Table S3).

Figure 7 shows that the rarefaction curves of phyllosphere fungal community for each plant sample did not show saturation. However, it also showed relatively high percent coverage ranging from 41.5% - 53.0% of Chao1 (Table 3). On the other hand, computation of Shannon index of diversity (H′) per plant revealed a wider range of value, where *Nephrolepis cordifolia*

showed highest with H′= 6.5 and *Beaucarnea recurvata* showed lowest with H′ = 2.8, but showed no significant differences (Table S6).

Drivers of fungal community structure - Beta diversity patterns

In order to determine the uniqueness of the associations of the phyllosphere fungal communities to room microclimate and plant host species, ordination analysis and ANOSIM were performed. BIO-ENV analysis was also done to define which abiotic and/or biotic variables have higher correlation with the dissimilarity of the communities of phyllosphere fungi using the Euclidean distance (Clarke and Ainsworth 1993).

Analysis using relative abundance-based (Bray-curtis dissimilarities) PCoA showed inconspicuous clustering of the fungal communities (Figure 8a and 8b). Phyllosphere fungal communities in the Temperate and Cold rooms were closer to each other, while communities from the Tropical, Nursery and Succulent rooms were closer to one another, albeit relatively scattered. ANOSIM results also showed significant distances in fungal composition between different rooms (*P=0.001, R=0.40*), indicating that, although weak, there was some association between room microclimate and phyllosphere fungal community composition.

On the other hand, ANOSIM showed relatively higher correlation of fungal community diversity to plant samples (*P=0.001, R=0.89*). Similar to our previous study on phyllosphere bacterial community, the correlation of the fungal community composition was observed associated to plant leaf shape and size. This time, however, the correlation was more stringent. It was observed that only those plants with remarkably similar leaf shape and size exhibited fungal communities that were clustered closer. This was observed in *Dracaena fragrans*, *Howea fosteriana*, and *Dracaena draco*; having a common linear, long, non-lobed, sword-like (ensiform) leaf-shape. *Beaucarnea recurvata* also exhibited the same ensiform leaf-shape but was narrower than the rest of these plant species; correspondingly it also showed distant clustering of fungal communities in comparison. The same observation was apparent in the two *Musa* species shared a common oblong, long, and wide leafshape. *Musa x paradisiaca*, however, exhibited a longer leaf size compared to *Musa acuminata*. Consequently, ordination analysis showed highly distant clusters of bacterial communities between the two *Musa* species.

BIO-ENV analysis provides further evidences of the stronger correlation between bacterial community and plant species. In Figure 9, the vectors represent the Spearman rank correlations

(ρs) between the abiotic and biotic factors influencing the distribution of the fungal community on the leaf surface of the greenhouse plants. According to BIO-ENV analysis, "Samples" (i.e. plant species) had strong influence on the bacterial population dynamics in the phyllosphere of the greenhouse plants, being the variable that explains the distribution of the relative abundance of the bacterial community (*BEST = 0.9205*).

Sample plants in the Tropical room best summarized the higher influence of plant species on the fungal community composition of the phyllosphere. Despite experiencing the same ambient climatic condition clustering of the fungal communities were highly inclined to plant species (Figure 8b). This species effect was also shown in Figure 9 where the vector representing "Sample" indicates its directional influence.

Antagonistic potential of phyllosphere fungal community against *B. cinerea*

A total of 629 fungal isolates from the phyllosphere of 14 greenhouse plants were screened for their antagonistic potential against *B. cinerea* using TCVA. Antagonistic effect observed includes inhibition of mycelial growth and spore germination (Figure 10).

Table 1 showed the number of isolates per plant sample and the percentage of fungal strains that tested positive for both antagonistic effects. *Olea europaea* showed the highest percentage of antagonistic fungal strains where 33% of the total fungal isolates exhibited inhibitory effect on both mycelial growth and spore germination of *B. cinerea*.

This study focused on identifying the 85 isolates that showed optimum antagonistic potential against the model pathogenic fungi. BOX-PCR fingerprinting and analysis, further divides these isolates into 39 genotypic groups at 60 % cut-off level. These groups was further observed consist of 3 different classes including *Eurotiomycetes*, *Dothideomycetes*, and *Tremellomycetes*. Using SANGER sequencing, 17 species were identified, out of these genotypic groups (Table 4). Frequently isolated antagonistic fungal species includes: *Penicillium brevicompactum* (10), *Penicillium polonicum* (5), *Cladsporium sphaerospermum* (5), and *Penicillium crustosum* (4). It was also noted that *Penecillium* was highly represented with 13 different species namely *P. brevicompactum, P. paxili, P. rubens, P. raistrickii, P. stecki, P. corylophilum, P. commune, P. adametzioides, P. restricum, crustosum, P. polonicum, P. copticola,* and *P. nothofagi*. Three days after the initial set-up, the VOCs produced by these isolates decreased the fungal colony

diameter by about 32.89% - 72.23% compared to the control; albeit ANOVA showed no significant differences between the inhibition percentages. (Table S7).

During the initial screening for antagonistic potential, it was also observed that a few fungal strains promote rather than inhibit the growth of *B. cinerea*. After thorough investigation, six fungal strains showed consistent growth promotion of *B. cinerea*; albeit very minimal and did not show significant differences (Table 5). BOX-PCR fingerprinting together with SANGER sequencing assigned these strain into six different species belonging to 4 different fungal classes. *Sarocladium strictum and Fusarium circinatum*; class *Sardariomycetes, Cladosporium sphaerospermum, and Cladosporium pini-ponderosae*; class *Dothideomycetes, Penicillium steckii*; class *Eurotiomycetes,* and *Cryptococcus flavescens*; class *Tremellomycetes*.

Discussion

Using cultivation-dependent and high throughput analysis, this study found high abundance and diversity of fungal community in the phyllosphere of the 14 greenhouse plants. It was also established that plants species have higher influence on fungal community composition, and plant leaf morphology was correlated to the distances in community composition of phyllosphere-associated fungi.

The documented fungal community of the phyllosphere (combining leaf samples from 14 different plant species) consists primarily, by members of the phylum *Ascomycota* and *Basidiomycota*. Fungal taxa identified were similar to those found in previous studies, including high relative abundance of *Capnodiales*, *Wallemiales*, and *Tremellales* (Jumpponen and Jones, 2009; Cordier et al, 2012; Bálint et al, 2013). The dominance of these fungal taxa in the overall phyllosphere community can be due in part to their association with the host plants since the distribution of the *Ascomycetous* and *Basideomycetous* fungal taxa was not the same for all plant samples, where some have higher abundance of *Ascomycota* while others have higher *Basidiomycota*. This association will be further discussed in the later part of this paper. The adaptive abilities of the members of these fungal taxa also facilitated for their dominance in the phyllosphere. The phyllosphere is considered a stressful environment partly because of exposure to UV radiation and fluctuating water availability (Lindow and Brandl, 2003; Whipps et al, 2008; Vorholt, 2012). Melanization and DNA repair have been established as UV-protection response from microorganisms inhabiting the phyllosphere (Grishkan, 2011; Fernandez et al, 2012). Melanins that are responsible for the dark-green, brown, and black color of fungi are also apparently responsible for adaptive properties that enable them to survive under conditions of environmental stress, such as osmotic extremes, UV radiation, and desiccation (Sterflinger, 2006).

Many melanized species belonging to order *Capnodiales* and *Pleosporales* have been isolated from environments highly exposed to UV radiation (Grishkan, 2011; Kembel and Mueller, 2014). This explains not only the dominance but also the ubiquitous nature of *Capnodiales* in the phyllosphere of the greenhouse plants observed. Aside from melanin, carotenoid pigments in yeasts has also been established to exhibit photoprotection (Moliné et al, 2010; Castiliani et al, 2014) and member of the order *Tremellales* that were identified in this study were similar to those that were previously characterized with carotenoid pigmentation (Inácio et al, 2005). This

study also identified yeasts that were reported to produce and accumulate mycosporines (Fernandez et al, 2012). This fungal metabolite is known for its photoprotective effect on yeasts protecting them from UVB-induced DNA damage (Moliné et al, 2011). Aside from UV-protection microbial phyllosphere colonists also showed adaptive mechanisms that counters the fluctuating water availability in the phyllosphere (Vorholt, 2012). Members of the order *Wallemiales* that were identified in this study were also reported to be xerophilic fungi (Zalar et al, 2006). The growth of these xerophilic fungi on artificial media proved to be independent to the solute used in order to lower water activity (Vaisey 1955; Pitt and Hocking 1977; as cited by Zalar et al, 2006). The abundance of the xerophilic fungi identified in this study suggests that this mechanism is also imperative for successful colonization of the phyllosphere.

It was mentioned earlier that plant-host and fungi association also accounts for the diversity of the fungal taxa observed in this study. Fungal communities inhabiting the phyllosphere of the 14 greenhouse plants vary within and across individual plants (Figures 3 and 4). Except for *Nerphrolephis cordifolia*, a higher variation of the fungal community was observed across than within plant species. This implies that different plant species affects the selection of respective fungal associates of their phyllosphere fungal community. The same influence was also observed in bacterial community composition of the same 14 greenhouse plant samples in our previous study on phyllosphere bacterial communities.

On the other hand, the high variation within the samples from *Nephrolephis cordifolia* may be attributed to the age of the plant. Originating from the Nursery room, *Nephrolephis cordifolia* is the only sample in this study that was relatively younger than the rest of the plants, and the high variability of associated fungi from within the leaf samples further supports previous reports that younger leaves harbors greater number of taxa than those of the older leaves (Ercolani, 1991; Thompson et al, 1993).

Plant species influence on the fungal community composition was made stronger by the variation observed in community composition within and across different greenhouse rooms. Fungal community composition was higher within than across different rooms, implying that different species found inside the same rooms harbors different fungal community composition. This was further supported by the results of BIO-ENV analysis where the BEST value was highest for “Samples” (plant samples) indicating that this factor best explains the distribution of the associated fungal community. Therefore, these evidences signify that plant species have

higher effect on the phyllosphere fungal community in comparison to the ambient room temperature.

The correlation between plant leaf morphology and distances of fungal community also further strengthens the evidences of higher plant-host influence on the phyllosphere fungal community composition. *Dracaena fragrans*, *Dracaena draco* and *Howea fosteriana*, exhibiting the same ensiform leaf-shape and leaf size, harbored fungal communities that are closer to one another. The same correlation was observed in our previous study on phyllosphere bacterial community, however the correlation of the distances of fungal community to plant-leaf morphology was more stringent. Along with *Dracaena fragrans*, *Dracaena draco* and *Howea fosteriana*, *Beaucarnea recurvata* exhibited closer bacterial community composition. This difference in fungal and bacterial community correlation to leaf morphology highlights the fact that the leaf characteristics that highly influenced the microbial community structure of the plant samples may be those that were not covered in this study. Nevertheless, these findings further supports previous reports that individual plants can have exclusive microbial associates possibly owing to their genetic makeup that ultimately controls their phenotypic characteristics and metabolism that is responsible for production of microbial attractants or defenses (Whipps, et al 2008; Hartmann et al. 2009; Cordier et al, 2012; Vorholt, 2012; Bálint et al, 2013; Kembel and Mueller, 2014).

Evidences that the phyllosphere fungal community of the samples plants consist of species that produces antifungal-volatiles were also presented in this study. Fungal species belonging to the genera *Penicillium*, *Cryptococcus*, and *Cladosporium* exhibited antagonistic effect to the pathogen *B. cinerea* by inhibiting its mycelial growth by approximately 30% to 80% with a visible reduction, if not total inhibition, of spore formation. The biocontrol activity of *Penicillium and Cryptococcus* against *B. cinerea* (Rouissi et al, 2013; Helbig 2001) and of *Cladosporium* as a mycoparasite has been well documented (Kiss, 2003) along with a comprehensive review on fungal volatiles produced by representative species from these genera (Morath et al, 2013).

Remarkably, it was also observed that VOCs produced by *Cladosporium sphaerospremum, Cladosporium pini-ponderosae* and *Penicillium steckii* have both negative and positive effects on the mycelial growth of *B. cinerea*. Apparently, fungal strains from the same lineage can have different bioactivity since they can differ significantly in their quantitative and qualitative secondary metabolite production (Dresch et al, 2015). Since fungal VOCs are produced during primary and secondary metabolism (Crespo et al, 2006) a strain-specific effect may have occurred causing the bipolar bioactivity of *Cladosporium sphaerospremum, Cladosporium*

piniponderosae and *Penicillium steckii* against *B. cinerea* in this study. Nevertheless, additional investigation, like strain selection along with bioactivity testing, is recommended to further explain the opposing bioactivity of these three fungal species.

The results of this study provide insights into the structure, variability in distribution, and antagonistic potential of phyllosphere fungal communities of plants grown inside a built environment. Similar to our previous study on phyllosphere bacterial community, the ambient room microclimate had little influence on the phyllosphere communities, and plant taxonomy and traits have higher correlation to the fungal community composition. This implies that plants have a stable fungal diversity composition regardless of the room microclimatic condition.

The implication of plant species having higher influences on the fungal community composition of their associated microbes can be beneficial in setting a healthy built environment that is ultimately favorable to human health. It can also be beneficial in maintaining a healthy indoor air quality of built environments, since both plants placed indoors (Orwell et al, 2004, Pegas et al, 2012) and their associated microbes work together in improving the air quality by absorbing, degrading, detoxifying, and sequestering air pollutants (Kim et al, 2008; Weyens et al, 2015). It also poses the possibility of limiting the growth of pathogenic molds and fungi, which are harmful to human health and can possibly cause "sick building" syndrome (Strauss, 2009), since the phyllosphere bacterial community also includes species that produces antifungalvolatiles. It presents the possibility of establishing the room microbiome by choosing plant species placed indoors (Mahnert et al, 2015) and possibly increase microbial diversity and beneficial microorganisms (Berg et al, 2014).

Comprehension of the driving forces of the microbial community structure is also imperative to the manipulation of plant-associated microbiome for the development of biocontrol methods that can be used as means of plant protection (Lindow and Brandl, 2003). Understanding the microbial structure of the target plant and utilization of microorganisms that occupy the same ecological niche as that of the plant pathogen can result to a more effective biocontrol that is less damaging to the environment.

Conclusion

An abundant and diverse fungal community inhabits the phyllosphere of 14 plants species grown under different controlled microclimate. Plant species strongly influence the distribution of the fungal communities as exhibited by the pronounced interspecies variation within and across different rooms and plant species. Base on plant leaf morphology, a correlation between the dissimilarity of fungal communities to the genetic distances of the plant species was established. The phyllosphere fungal community also includes VOCs-producing species antagonistic to both the mycelial growth and spore germination of the pathogenic fungi *B. cinerea*. Along with these antagonists, some species were also found to have bipolar bioactivity against the pathogenic fungi.

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Figures and Tables for:

Plant-host taxonomy and genotype as drivers of fungal community structure and the antagonistic potential of fungi on the leaves of greenhouse plants

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Table 1. Plate counts (CFU/cm^{-2}) and percentages of antagonistic fungi against the plant pathogenic fungi *Botrytis cinerea*.

Greenhouse room	Plant sample	CFU ± SD		Total isolates tested	Inhibits growth		Inhibits sporulation		Inhibits both growth and sporulation	
		SNA	Sab		No.	%	No.	%	No.	%
Tropical	*Aechmea eurycorymbus*	[a]4.44E+05 ± 7.00E+04	[a]1.05E+05 ± 1.47E+04	78	2	3	42	54	2	3
	Dracaena marginata	[a]2.95E+05 ± 4.20E+04	[a]3.73E+05 ± 9.51E+04	78	2	3	46	59	2	3
	Epipremnum aureum	[a]1.23E+05 ± 1.16E+05	[a]6.63E+04 ± 5.94E+04	29	3	10	21	72	2	7
	Musa paradisiaca	[b]1.07E+06 ± 1.59E+05	[b]7.32E+05 ± 8.51E+04	77	13	17	37	48	13	17
Temperate	*Dracaena fragrans*	[a]1.45E+04 ± 6.68E+03	[a]2.26E+04 ± 1.27E+04	16	4	25	9	56	4	25
	Howea forsteriana	[a]3.36E+04 ± 9.81E+03	[a]3.58E+04 ± 1.30E+04	24	6	25	14	58	5	21
	Malvaviscus penduliflorus	[a]5.88E+03 ± 3.40E+03	[a]6.95E+03 ± 1.94E+03	12	1	8	8	67	0	0
Nursery	*Nephrolepis cordifolia*	[a]1.43E+03 ± 1.17E+03	[a]1.69E+03 ± 1.37E+03	24	4	17	15	63	3	13
Cold	*Chlorophytum comosum*	[a]1.81E+05 ± 7.76E+04	[a]1.94E+05 ± 8.28E+04	79	9	11	50	63	9	11
	Dracaena draco	[a]2.27E+04 ± 5.68E+03	[a]3.96E+04 ± 1.14E+04	21	5	24	13	62	5	24
	Olea europaea	[a]2.37E+04 ± 4.23E+03	[a]3.84E+04 ± 2.93E+03	18	6	33	12	67	6	33
Succulent	*Aloe arborescens*	[a]2.81E+04 ± 8.02E+03	[a]2.86E+04 ± 5.21E+03	53	16	30	23	43	15	28
	Beaucarnea recurvata	[a]6.03E+03 ± 1.18E+03	[a]3.31E+04 ± 1.58E+04	74	11	15	19	26	10	14
	Musa acuminata	[a]9.84E+02 ± 6.94E+02	[a]4.50E+02 ± 1.46E+02	46	10	22	12	26	9	20

**Means that do not share the same letter are significantly different (alpha=0.05).*

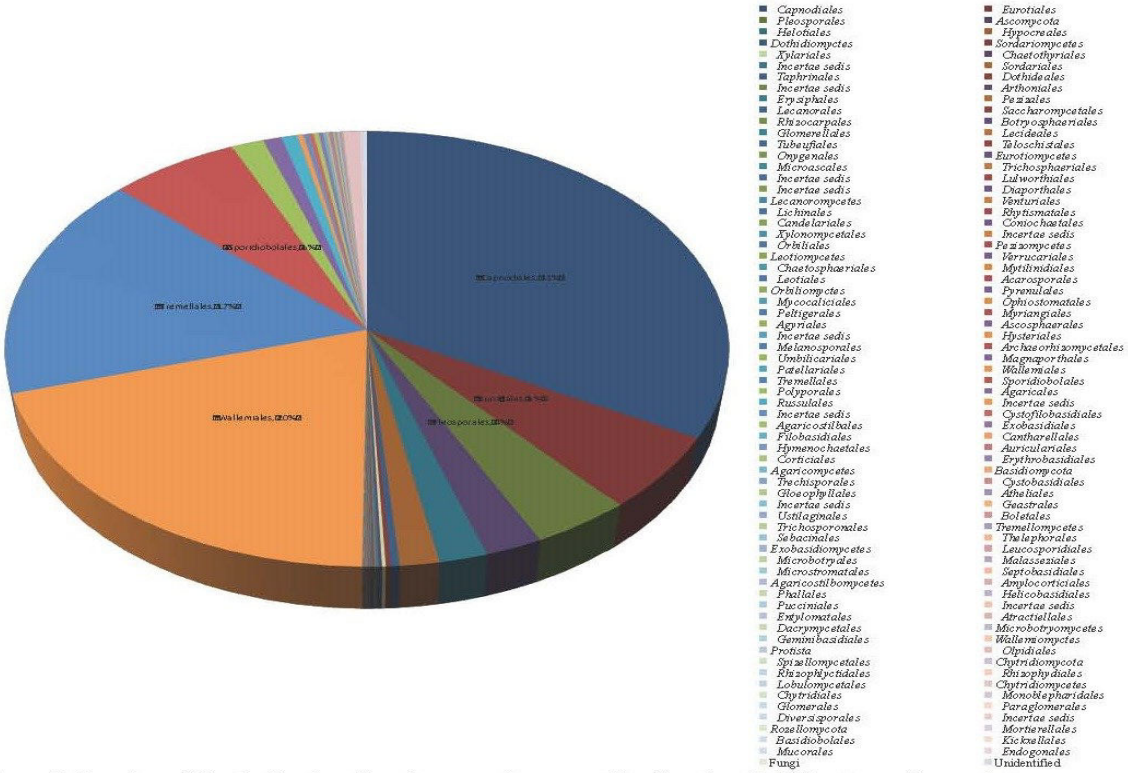

Figure 1. Overview of the phyllosphere fungal community composition found on 14 different greenhouse plants.

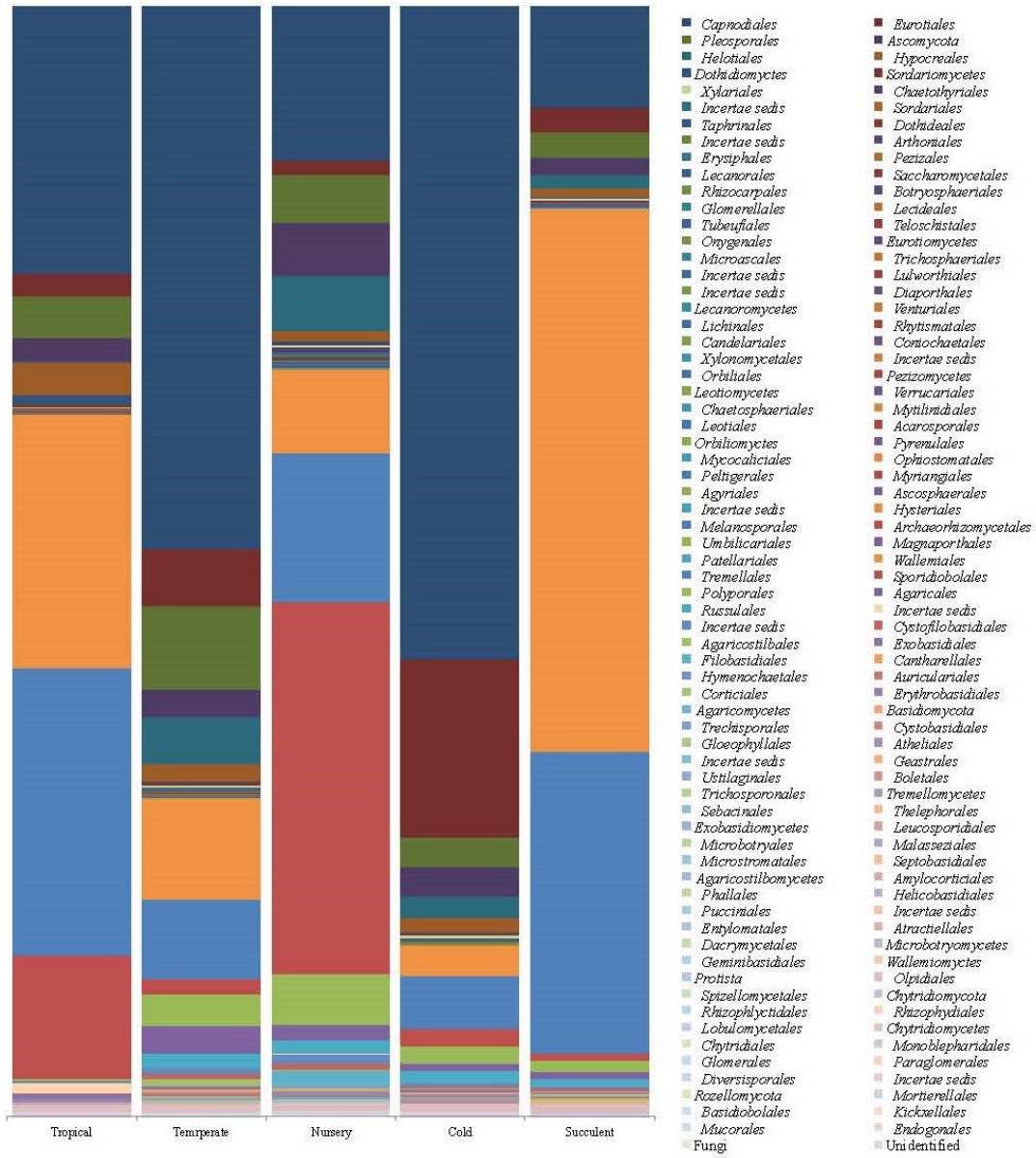

Figure 2. Relative abundance of phyllosphere fungal communities composition found on 14 different greenhouse plants categorized per greenhouse room.

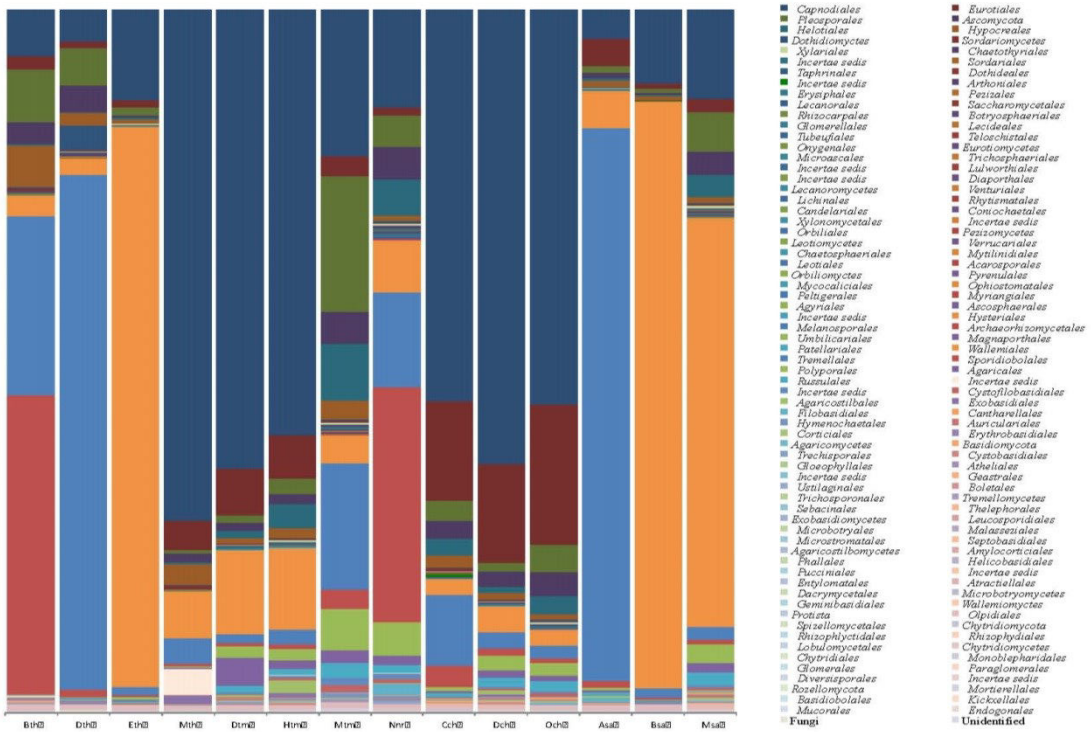

Figure 3. Relative abundance of phyllosphere fungal community composition found on 14 different greenhouse plants categorized per plant species.

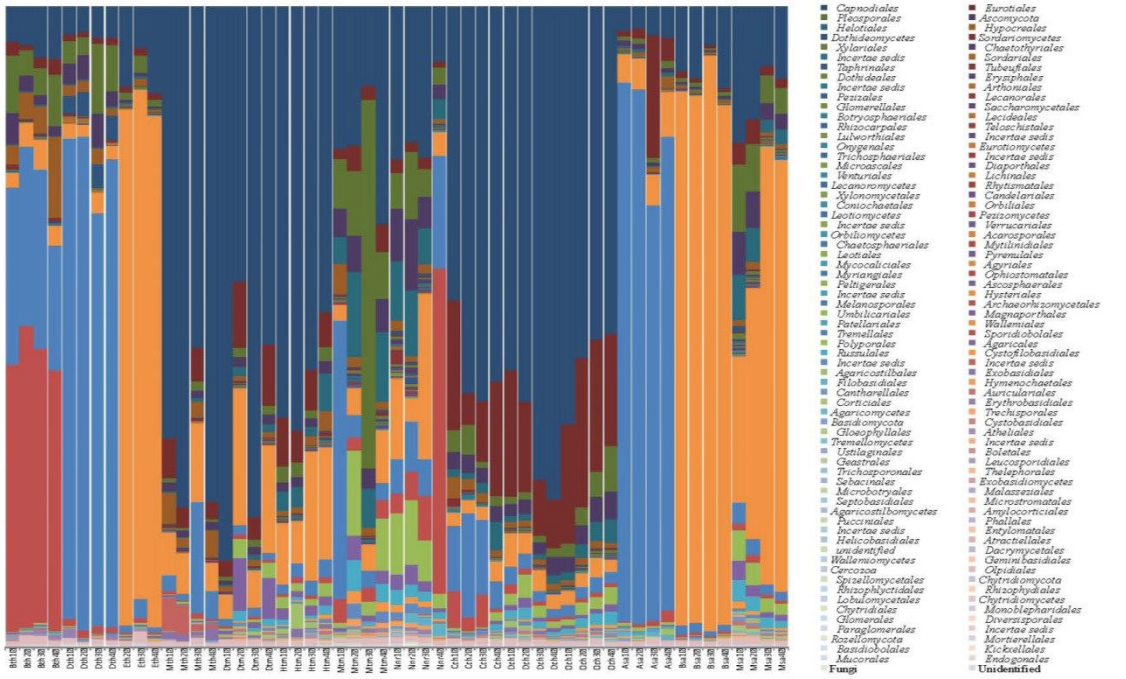

Figure 4. Relative abundance of phyllosphere fungal community composition from four different replicates of each14 different greenhouse plants.

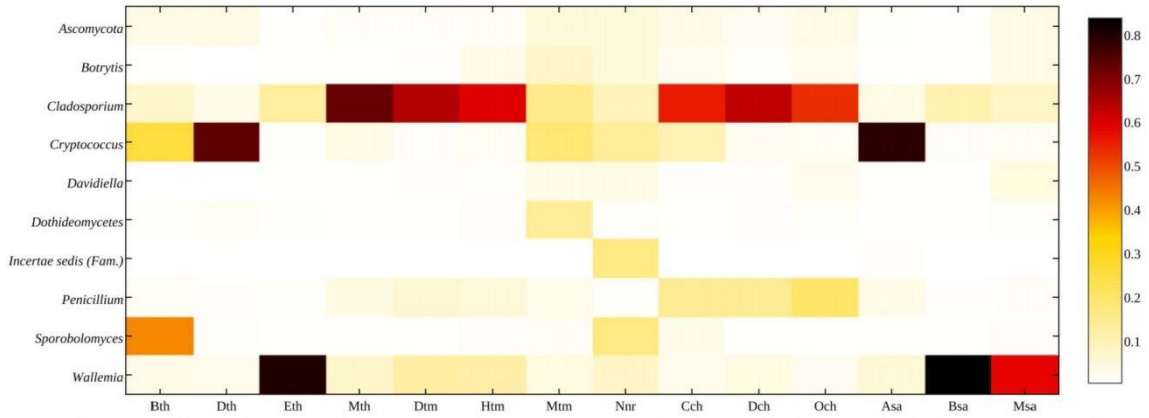

Figure 5. Relative abundance of dominant fungal genera (cut-off 1%) found on the phyllosphere of 14 different greenhouse plants.

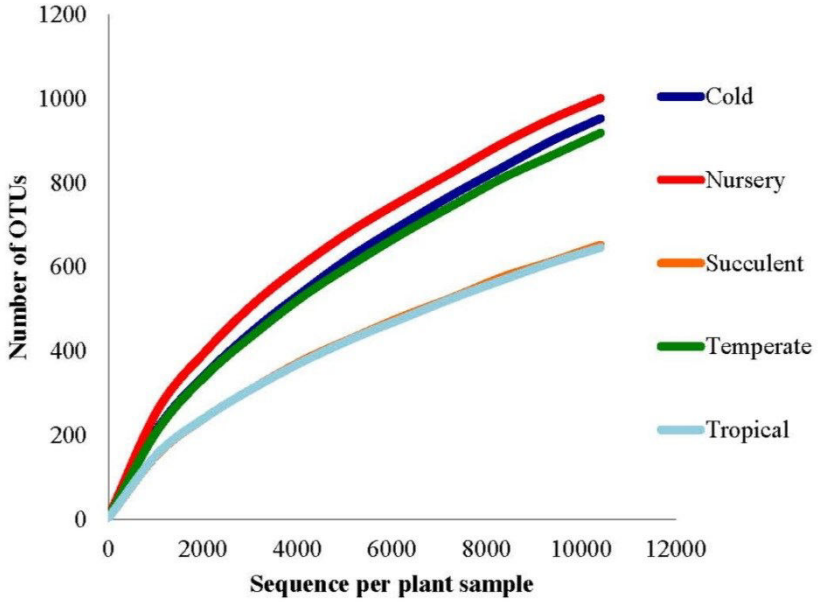

Figure 6. Rarefaction results for the diversity of phyllosphere fungal community of 14 greenhouse plants. Diversity per room is represented.

Table 2. Species richness estimate (categorized per room) obtained at 3% genetic dissimilarity using Miseq Illumina-derived sequences of the DNA extracted from 14 greenhouse plants.

Greenhouse room	Seqs/Sample	Shannon index (H')	Observed species (no. of OTUs)	Chao1 (no. of OTUs)	Coverage (%)
Cold	10410	5.3	953.0	2056.4	46.3
Nursery	10410	6.5	1000.8	1887.4	53.0
Succulent	10410	4.2	652.6	1418.2	46.0
Temperate	10410	5.4	918.2	1919.1	47.8
Tropical	10410	4.4	646.1	1409.1	45.8

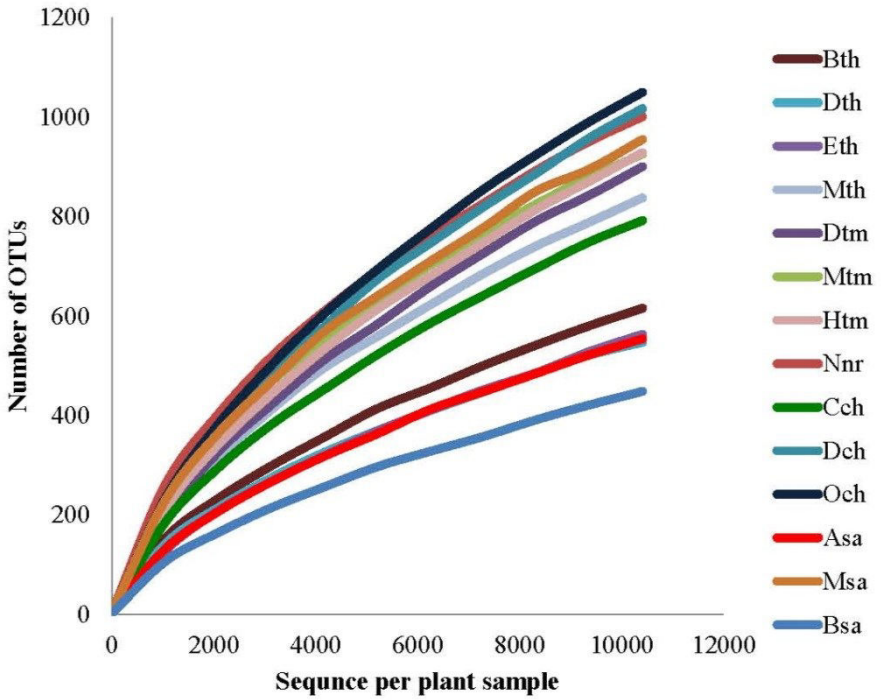

Figure 7. Rarefaction results for the diversity of phyllosphere fungal community of plants. Diversity per plant is represented

Table 3. Species richness estimate (categorized per plant) obtained at 3% genetic dissimilarity using Miseq Illumina-derived sequences of the DNA extracted from 14 greenhouse plants.

Greehouse room	Sample	Origin	Seqs/Sample	Shannon index (H')	Observed species (no. of OTUs)	Chao1 (no. of OTUs)	Coverage (%)
Tropical	Bth	*Aechmea eurycorymbus*	10410	4.4	615.5	1406.6	43.8
	Dth	*Dracaena marginata*	10410	4.5	547.1	1163.9	47.0
	Eth	*Epipremnum aureum*	10410	3.2	563.2	1358.7	41.5
	Mth	*Musa paradisiaca*	10410	5.0	837.7	1694.6	49.4
Temperate	Dtm	*Dracaena fragrans*	10410	4.9	900.4	1974.1	45.6
	Htm	*Howea forsteriana*	10410	5.3	928.7	1979.3	46.9
	Mtm	*Malvaviscus penduliflorus*	10410	6.1	925.6	1804.0	51.3
Nursery	Nnr	*Nephrolepis cordifolia*	10410	6.5	1000.8	1887.4	53.0
Cold	Cch	*Cch comosum*	10410	5.0	791.9	1753.6	45.2
	Dch	*Dracaena draco*	10410	5.5	1017.1	2190.8	46.4
	Och	*Oleaeuropaea*	10410	5.4	1050.0	2224.7	47.2
Succulent	Asa	*Aloe arborescens*	10410	4.1	554.2	1315.6	42.1
	Bsa	*Beaucarnea recurvata*	10410	2.8	448.5	1052.9	42.6
	Msa	*Musa acuminata*	10410	5.5	955.0	1886.1	50.6

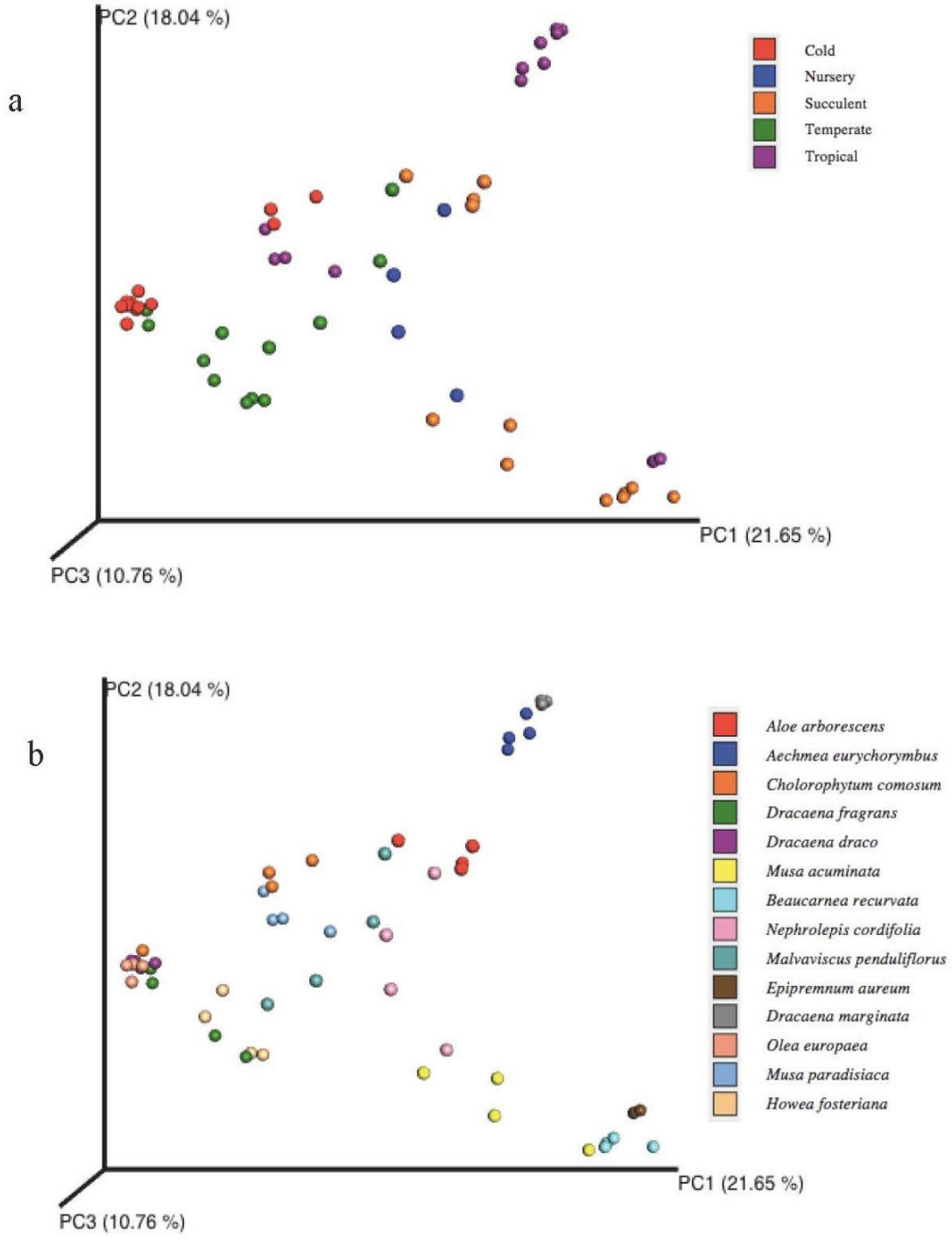

Figure 8. PCoA plots showing the clustering patterns between samples in A) greenhouse rooms, and B) plant species, based on Bray-Curtis dissimilarity.

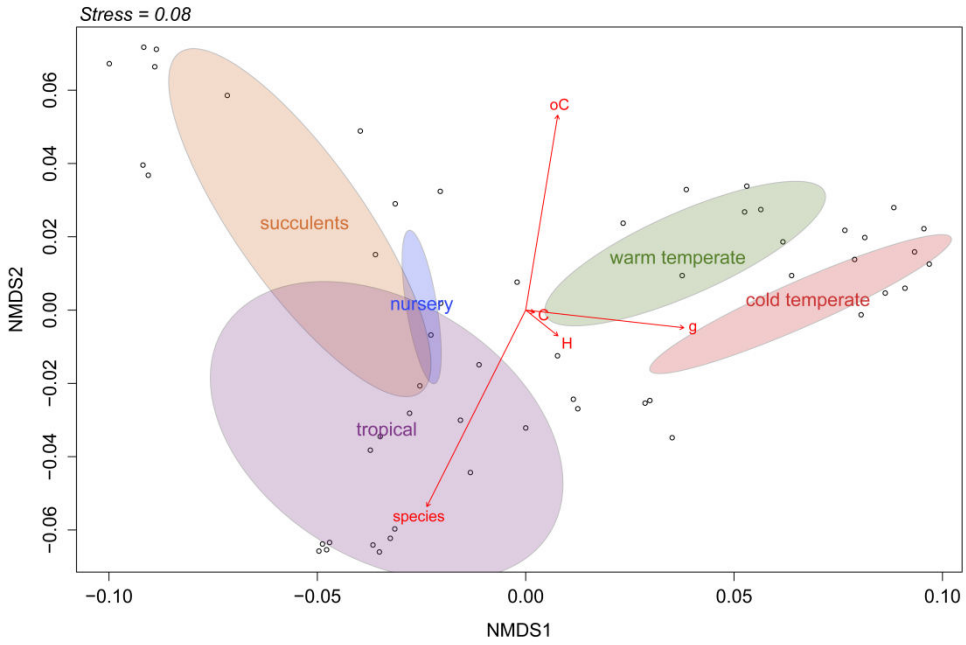

Figure 9. Non-metric multidimensional scaling (NMDS) plot derived from Bray-Curtis dissimilarity illustrating distances between fungal community compositions. The BIO-ENV vectors of environmental variables based on Euclidean distances represent the direction along the samples of each greenhouse rooms, showing the role each of them played in explaining the distribution of the samples and its directional influence.

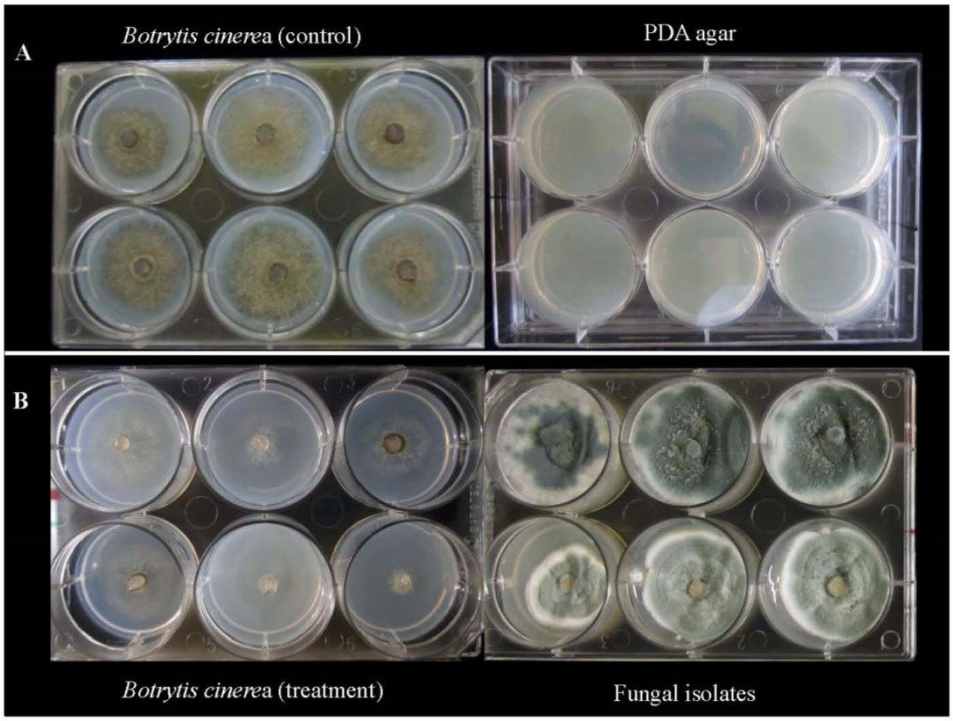

Figure 10. Two-clamp VOCs assay of fungal isolates from 14 greenhouse plants showing antifungal volatile activity against *Botrytis cinerea*. (A) *B. cinerea* plugs showing mycelial growth and spore germination (left), paired with an empty PDA plate (right). (B) Volatile organic compounds produced by fungi (right) affected mycelial growth and germination of spores (left), compared to the control (above left).

Table 4. Taxonomic classification of fungal strains with antagonistic effect against *Botrytis cinerea* isolated from the phyllosphere of 14 greenhouse plants.

Greenhouse room	Strains	Plant of origin	Score	Species	Ident (%)	Accession
Tropical	Bth3Sab1	*Aechmea eurycorymbus*	++	*Penicillium brevicompactum* NRRL 2011	97	NR_121299.1
	Dth2Sab10	*Dracaena marginata*	++	*Penicillium paxilli* CBS 360.48	99	NR_111483.1
	Eth1Sab2	*Epipremnum aureum*	++	*Penicillium rubens* CBS 129667	99	NR_111815.1
	Eth4Sab3		++	*Cladosporium sphaerospermum* CBS 193.54	100	NR_111222.1
	Mth3Sab3	*Musa paradisiaca*	++	*Penicillium brevicompactum* NRRL 2011	97	NR_121299.1
	Mth4Sab3		++	*Penicillium raistrickii* FRR 1044	100	NR_119493.1
	Mth4Sab9		++	*Penicillium steckii* CBS 260.55	99	NR_111488.1
Temperate	Dtm3Sab6	*Dracaena fragrans*	++	*Penicillium brevicompactum* NRRL 2011	99	NR_121299.1
	Dtm4SNA9		++	*Penicillium brevicompactum* NRRL 2011	99	NR_121299.1
	Dtn3SNA1		++	*Penicillium corylophilum* NRRL 802	99	NR_121236.1
	Htm4Sab2	*Howea forsteriana*	++	*Penicillium commune* CBS 311.48	99	NR_111143.1
	Htm4SNA7		+++	*Penicillium adametzioides* CBS 313.59	100	NR_103660.1
Nursery	Nnr4SNA1	*Nephrolepis cordifolia*	++	*Penicillium restrictum* NRRL 1748	98	NR_121239.1
Cold	Cch1Sab4	*Chlorophytum comosum*	++	*Penicillium crustosum* FRR 1669	99	NR_077153.1
	Cch1SNA2		++	*Penicillium polonicum* CBS 222.28	99	NR_103687.1
	Cch1SNA3		++	*Penicillium brevicompactum* NRRL 2011	99	NR_121299.1
	Cch3SNA8		+++	*Penicillium polonicum* CBS 222.28	100	NR_103687.1
	Cch4SNA8		+++	*Cladosporium sphaerospermum* CBS 193.54	100	NR_111222.1
	Dch3Sab5	*Dracaena draco*	+++	*Penicillium polonicum* CBS 222.28	100	NR_103687.1
	Dch3Sab8		++	*Penicillium polonicum* CBS 222.28	100	NR_103687.1
	Dch4Sab2		++	*Penicillium polonicum* CBS 222.28	99	NR_103687.1
	Och3SNA6	*Olea europaea*	++	*Penicillium brevicompactum* NRRL 2011	99	NR_121299.1

[a]*Inhibition score (+) indicates 10-30%; (++) 31-60%; (+++) 61-80%; (++++) 81-100% mycelial growth inhibition on Botrytis cinerea.*

Table 4. cont.

Greenhouse room	Strains	Plant of origin	Score	Species	Ident (%)	Accession
Cold	Och4Sab4		+++	*Penicillium crustosum* FRR 1669	99	NR_077153.1
Succulent	Asa1SNA1	*Aloe arborescens*	+++	*Cryptococcus magnus* CBS 140	99	NR_130655.1
	Asa3SNA1		+++	*Penicillium brevicompactum* NRRL 2011	99	NR_121299.1
	Asa3SNA10		+++	*Penicillium brevicompactum* NRRL 2011	99	NR_121299.1
	Asa4Sab9		++	*Penicillium copticola* CBS 127355	99	NR_121516.1
	Asa4SNA6		+++	*Cryptococcus albidus* CBS 142	99	NR_111046.1
	Bsa2Sab3	*Beaucarnea recurvata*	+++	*Penicillium brevicompactum* NRRL 2011	97	NR_121299.1
	Bsa2Sab7		++	*Penicillium crustosum* FRR 1669	99	NR_077153.1
	Bsa2SNA4		+++	*Cladosporium sphaerospermum* CBS 193.54	99	NR_111222.1
	Bsa4Sab5		++	*Cladosporium pini-ponderosae*	97	NR_119730.1
	Bsa4SNA3		++	*Penicillium brevicompactum* NRRL 2011	99	NR_121299.1
	Bsa4SNA6		++	*Cladosporium sphaerospermum* CBS 193.54	100	NR_111222.1
	Msa2Sab7	*Musa acuminata*	++	*Penicillium nothofagi* CBS 130383	99	NR_121518.1
	Msa2SNA5		++	*Cladosporium sphaerospermum* CBS 193.54	100	NR_111222.1
	Msa3Sab6		++	*Cladosporium pini-ponderosae*	100	NR_119730.1
	Msa3SNA4		++	*Penicillium crustosum* FRR 1669	99	NR_077153.1

[a]*Inhibition score (+) indicates 10-30%; (++) 31-60%; (+++) 61-80%; (++++) 81-100% mycelial growth inhibition on Botrytis cinerea.*

Table S1. ANOVA of phyllosphere fungal CFU of 14 different greenhouse plants

Source of Variation	SS	df	MS	F	p-level	F crit
Between Groups	1.5455E+12	13	1.18885E+11	13.84069	0.00001	2.50726
Within Groups	1.20253E+11	14	8.58951E+09			
Total	1.66575E+12	27				

Table S2. Tukey's test of phyllosphere fungal CFU of 14 different greenhouse plants

	Plat samples	N	Subset for alpha 0.05	
			1	2
Tukey's HSD[a]	Msa	2	717.00	
	Nnr	2	1560.00	
	Mtm	2	6414.00	
	Dtm	2	18537.00	
	Bsa	2	19566.00	
	Asa	2	28368.00	
	Och	2	31050.00	
	Dch	2	31137.00	
	Htm	2	34677.00	
	Eth	2	94566.00	
	Cch	2	187765.50	
	Bth	2	274440.00	
	Dth	2	333900.00	
	Mth	2		901440.00
	Sig.		0.10	1.00

Means for groups in homogeneous subsets are displayed

a. Uses Harmonic Mean Sample Size = 2.0

Table S3. Alpha diversity statistical analysis of the Shannon index per greenhouse room

Group1	Group2	Group1 mean	Group1 std	Group2 mean	Group2 std	t stat	p-value
Cold	Tropical	5.29	0.43	4.37	0.71	3.82	0.02
Cold	Temperate	5.29	0.43	5.44	0.90	-0.50	1.00
Cold	Succulent	5.29	0.43	4.16	1.29	2.75	0.08
Cold	Nursery	5.29	0.43	6.53	0.85	-3.55	0.04
Nursery	Temperate	6.53	0.85	5.44	0.90	1.98	0.73
Nursery	Tropical	6.53	0.85	4.37	0.71	4.91	0.01
Nursery	Succulent	6.53	0.85	4.16	1.29	3.20	0.07
Temperate	Succulent	5.44	0.90	4.16	1.29	2.70	0.13
Tropical	Succulent	4.37	0.71	4.16	1.29	0.52	1.00
Tropical	Temperate	4.37	0.71	5.44	0.90	-3.33	0.04

Table S4. Alpha diversity statistical analysis of the Observed Species per greenhouse room

Group1	Group2	Group1 mean	Group1 std	Group2 mean	Group2 std	t stat	p-value
Cold	Tropical	952.98	153.10	646.05	129.87	5.42	0.01
Cold	Temperate	952.98	153.10	918.24	114.36	0.60	1.00
Cold	Succulent	952.98	153.10	652.56	242.09	3.48	0.02
Cold	Nursery	952.98	153.10	1000.78	136.01	-0.52	1.00
Nursery	Temperate	1000.78	136.01	918.24	114.36	1.11	1.00
Nursery	Tropical	1000.78	136.01	646.05	129.87	4.55	0.01
Nursery	Succulent	1000.78	136.01	652.56	242.09	2.56	0.29
Temperate	Succulent	918.24	114.36	652.56	242.09	3.29	0.05
Tropical	Succulent	646.05	129.87	652.56	242.09	-0.09	1.00
Tropical	Temperate	646.05	129.87	918.24	114.36	-5.49	0.01

Table S5. Alpha diversity statistical analysis of the Chao1 per greenhouse room

Group1	Group2	Group1 mean	Group1 std	Group2 mean	Group2 std	t stat	p-value
Cold	Tropical	2056.37	288.44	1409.11	231.95	6.22	0.01
Cold	Temperate	2056.37	288.44	1919.12	165.13	1.37	1.00
Cold	Succulent	2056.37	288.44	1418.20	379.58	4.44	0.01
Cold	Nursery	2056.37	288.44	1887.44	199.33	1.02	1.00
Nursery	Temperate	1887.44	199.33	1919.12	165.13	-0.29	1.00
Nursery	Tropical	1887.44	199.33	1409.11	231.95	3.57	0.05
Nursery	Succulent	1887.44	199.33	1418.20	379.58	2.21	0.48
Temperate	Succulent	1919.12	165.13	1418.20	379.58	4.01	0.02
Tropical	Succulent	1409.11	231.95	1418.20	379.58	-0.07	1.00
Tropical	Temperate	1409.11	231.95	1919.12	165.13	-6.18	0.01

Table S6. Alpha diversity statistical analysis of the Shannon index per plant sample

Group1	Group2	Group1 mean	Group1 std	Group2 mean	Group2 std	t stat	p-value
Asa	Dtm	4.141	0.635	4.920	0.631	-1.506	1.00
Asa	Cch	4.141	0.635	4.978	0.410	-1.919	1.00
Bsa	Dch	2.827	0.276	5.489	0.160	-14.432	1.00
Bsa	Cch	2.827	0.276	4.978	0.410	-7.536	1.00
Bsa	Asa	2.827	0.276	4.141	0.635	-3.284	1.00
Bsa	Mth	2.827	0.276	5.013	0.534	-6.290	1.00
Bsa	Dtm	2.827	0.276	4.920	0.631	-5.259	1.00
Bth	Dtm	4.394	0.256	4.920	0.631	-1.336	1.00
Bth	Dch	4.394	0.256	5.489	0.160	-6.278	1.00
Bth	Cch	4.394	0.256	4.978	0.410	-2.093	1.00
Bth	Asa	4.394	0.256	4.141	0.635	0.641	1.00
Bth	Mth	4.394	0.256	5.013	0.534	-1.808	1.00
Bth	Bsa	4.394	0.256	2.827	0.276	7.202	0.82
Cch	Dtm	4.978	0.410	4.920	0.631	0.135	1.00
Dch	Dtm	5.489	0.160	4.920	0.631	1.514	1.00
Dch	Asa	5.489	0.160	4.141	0.635	3.565	1.00
Dch	Cch	5.489	0.160	4.978	0.410	2.011	1.00
Dth	Mth	4.548	0.204	5.013	0.534	-1.407	1.00
Dth	Nnr	4.548	0.204	6.526	0.852	-3.911	1.00
Dth	Cch	4.548	0.204	4.978	0.410	-1.628	1.00
Dth	Bth	4.548	0.204	4.394	0.256	0.812	1.00
Dth	Bsa	4.548	0.204	2.827	0.276	8.673	1.00
Dth	Dch	4.548	0.204	5.489	0.160	-6.284	1.00
Dth	Asa	4.548	0.204	4.141	0.635	1.056	1.00
Dth	Dtm	4.548	0.204	4.920	0.631	-0.971	1.00
Dth	Och	4.548	0.204	5.400	0.454	-2.965	1.00
Eth	Dth	3.241	0.307	4.548	0.204	-5.705	1.00
Eth	Htm	3.241	0.307	5.333	0.253	-8.346	1.00
Eth	Mth	3.241	0.307	5.013	0.534	-4.343	1.00
Eth	Nnr	3.241	0.307	6.526	0.852	-5.386	1.00
Eth	Dtm	3.241	0.307	4.920	0.631	-3.587	1.00
Eth	Bsa	3.241	0.307	2.827	0.276	1.580	1.00
Eth	Cch	3.241	0.307	4.978	0.410	-5.204	1.00
Eth	Bth	3.241	0.307	4.394	0.256	-4.571	1.00
Eth	Dch	3.241	0.307	5.489	0.160	-10.599	1.00
Eth	Och	3.241	0.307	5.400	0.454	-6.004	1.00
Eth	Asa	3.241	0.307	4.141	0.635	-1.912	1.00
Eth	Mtm	3.241	0.307	6.066	1.139	-3.535	1.00
Htm	Bsa	5.333	0.253	2.827	0.276	11.588	1.00
Htm	Mth	5.333	0.253	5.013	0.534	0.939	1.00

Table S6. Cont.

Group1	Group2	Group1 mean	Group1 std	Group2 mean	Group2 std	t stat	p-value
Htm	Asa	5.333	0.253	4.141	0.635	3.021	1.00
Htm	Och	5.333	0.253	5.400	0.454	-0.224	1.00
Htm	Nnr	5.333	0.253	6.526	0.852	-2.325	1.00
Htm	Dch	5.333	0.253	5.489	0.160	-0.902	1.00
Htm	Cch	5.333	0.253	4.978	0.410	1.277	1.00
Htm	Dtm	5.333	0.253	4.920	0.631	1.053	1.00
Htm	Bth	5.333	0.253	4.394	0.256	4.520	1.00
Htm	Dth	5.333	0.253	4.548	0.204	4.187	1.00
Msa	Bth	5.511	0.961	4.394	0.256	1.944	1.00
Msa	Dtm	5.511	0.961	4.920	0.631	0.890	1.00
Msa	Cch	5.511	0.961	4.978	0.410	0.883	1.00
Msa	Dth	5.511	0.961	4.548	0.204	1.697	1.00
Msa	Och	5.511	0.961	5.400	0.454	0.180	1.00
Msa	Mtm	5.511	0.961	6.066	1.139	-0.646	1.00
Msa	Nnr	5.511	0.961	6.526	0.852	-1.369	1.00
Msa	Mth	5.511	0.961	5.013	0.534	0.784	1.00
Msa	Asa	5.511	0.961	4.141	0.635	2.059	1.00
Msa	Htm	5.511	0.961	5.333	0.253	0.309	1.00
Msa	Eth	5.511	0.961	3.241	0.307	3.331	1.00
Msa	Bsa	5.511	0.961	2.827	0.276	4.647	1.00
Msa	Dch	5.511	0.961	5.489	0.160	0.039	1.00
Mth	Dtm	5.013	0.534	4.920	0.631	0.195	1.00
Mth	Cch	5.013	0.534	4.978	0.410	0.089	1.00
Mth	Dch	5.013	0.534	5.489	0.160	-1.478	1.00
Mth	Asa	5.013	0.534	4.141	0.635	1.819	1.00
Mtm	Bsa	6.066	1.139	2.827	0.276	4.786	1.00
Mtm	Asa	6.066	1.139	4.141	0.635	2.557	1.00
Mtm	Mth	6.066	1.139	5.013	0.534	1.450	1.00
Mtm	Htm	6.066	1.139	5.333	0.253	1.088	1.00
Mtm	Bth	6.066	1.139	4.394	0.256	2.481	1.00
Mtm	Cch	6.066	1.139	4.978	0.410	1.557	1.00
Mtm	Dch	6.066	1.139	5.489	0.160	0.869	1.00
Mtm	Dtm	6.066	1.139	4.920	0.631	1.525	1.00
Mtm	Och	6.066	1.139	5.400	0.454	0.941	1.00
Mtm	Nnr	6.066	1.139	6.526	0.852	-0.560	1.00
Mtm	Dth	6.066	1.139	4.548	0.204	2.273	1.00
Nnr	Dch	6.526	0.852	5.489	0.160	2.072	1.00
Nnr	Bsa	6.526	0.852	2.827	0.276	7.151	1.00
Nnr	Bth	6.526	0.852	4.394	0.256	4.150	1.00
Nnr	Asa	6.526	0.852	4.141	0.635	3.887	1.00

Table S6. Cont.

Group1	Group2	Group1 mean	Group1 std	Group2 mean	Group2 std	t stat	p-value
Nnr	Mth	6.526	0.852	5.013	0.534	2.606	1.00
Nnr	Och	6.526	0.852	5.400	0.454	2.019	1.00
Nnr	Dtm	6.526	0.852	4.920	0.631	2.624	1.00
Nnr	Cch	6.526	0.852	4.978	0.410	2.835	1.00
Och	Mth	5.400	0.454	5.013	0.534	0.957	1.00
Och	Bsa	5.400	0.454	2.827	0.276	8.380	1.00
Och	Dtm	5.400	0.454	4.920	0.631	1.071	1.00
Och	Dch	5.400	0.454	5.489	0.160	-0.319	1.00
Och	Bth	5.400	0.454	4.394	0.256	3.342	1.00
Och	Cch	5.400	0.454	4.978	0.410	1.195	1.00
Och	Asa	5.400	0.454	4.141	0.635	2.793	1.00

Table S7. ANOVA of the percent inhibition of antagonistic fungal strains against the growth of *Botrytis cinerea*

Source of Variation	SS	df	MS	F	p-level	F crit
Between Groups	15.6509	38	0.41187	1.35598	0.11074	1.50748
Within Groups	35.5375	117	0.30374			
Total	51.1884	155				

Table S8. ANOVA of phyllosphere fungal CFU of sample plants from the Succulent room

Source of Variation	SS	df	MS	F	p-level	F crit
Between Groups	798,225,204	2	399,112,602	3.26523	0.17661	9.55209
Within Groups	366,693,138	3	122,231,046			
Total	1,164,918,342	5				

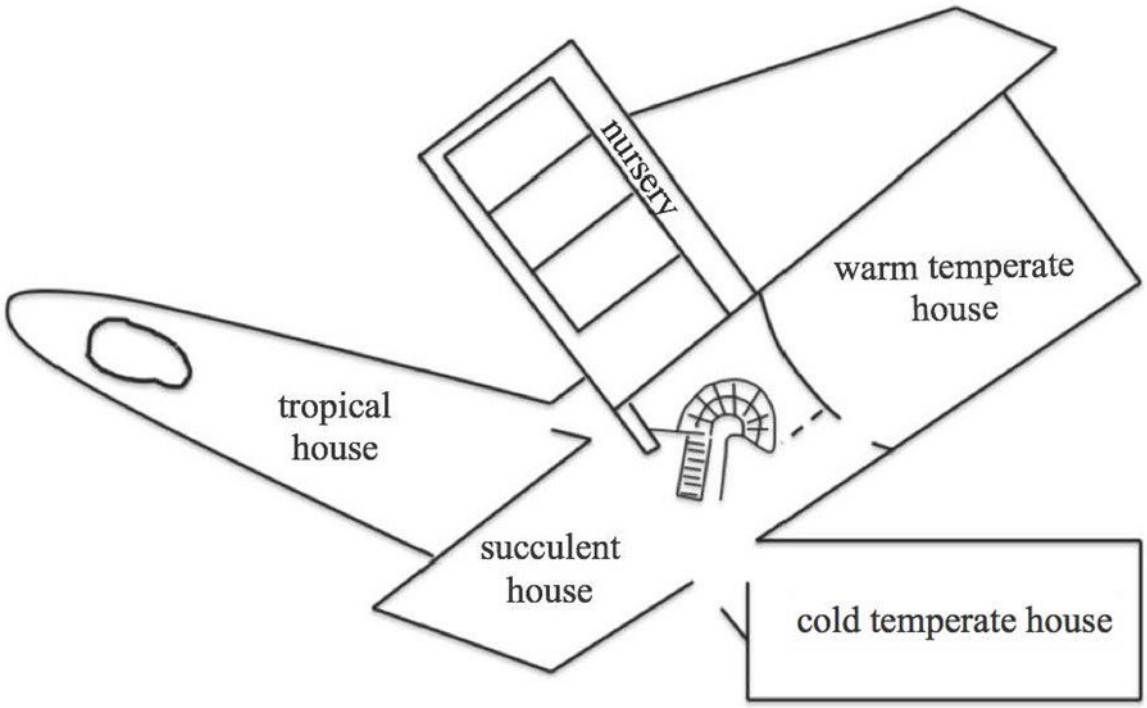

Figure S1. Diagram of the Botanical Garden of Graz greenhouse complex

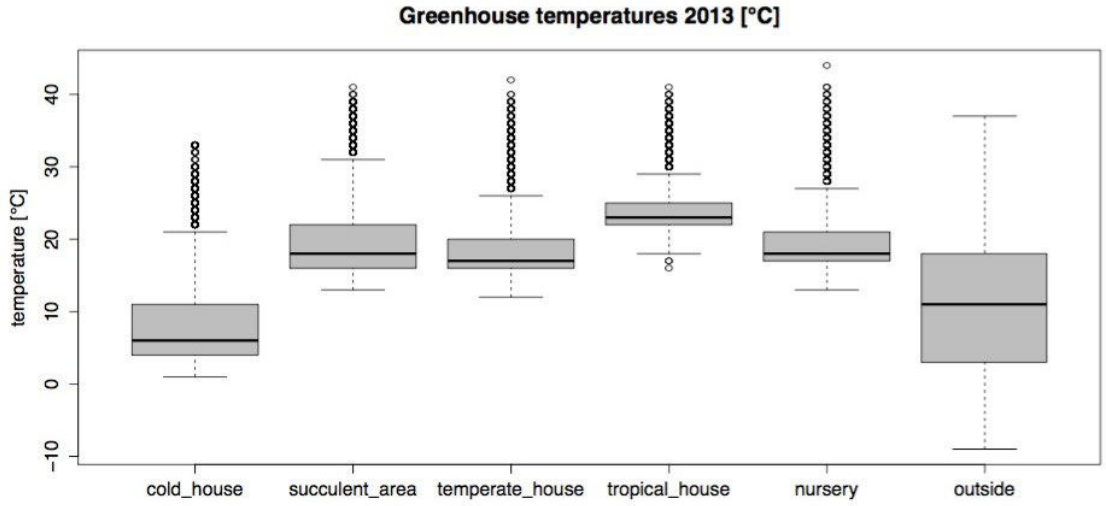

Figure S2. Mean temperature inside each room of the greenhouse complex for the year 2013

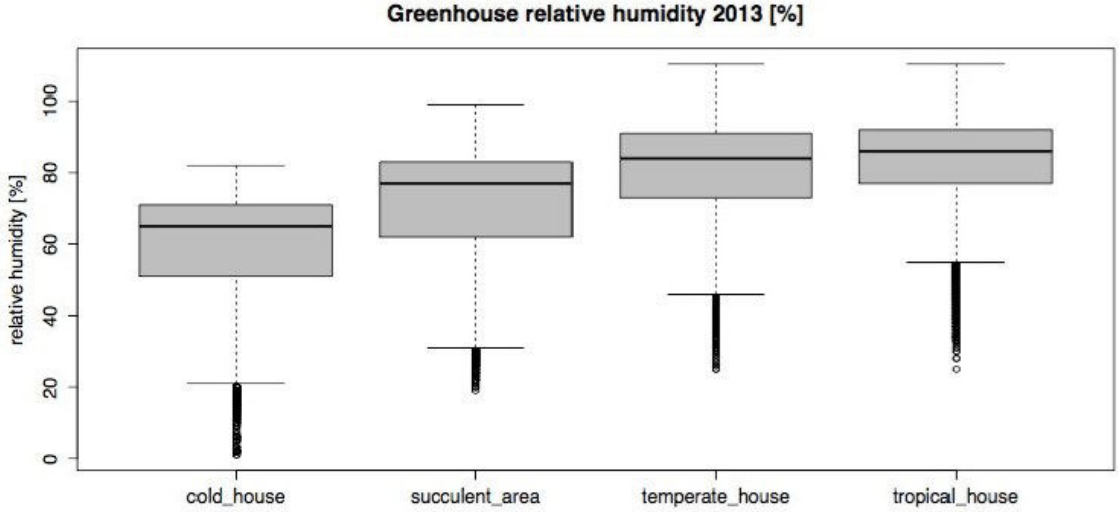

Figure S3. Mean humidity inside each room of the greenhouse complex for the year 2013

The altered microbiome of confined built environments

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Running title: Comparative metagenomics of controlled and uncontrolled built environments

Keywords: controlled and uncontrolled indoor microbiomes, cleanrooms, intensive care units, public buildings, private houses, wildlife park, shotgun metagenomics, 16S rRNA gene amplicons, viability

Abstract

Understanding the complex influences of human maintenance, environmental confinement and microbial control in built environments is critical to increase our health inside buildings. However, to date the scientific community lacks clear concepts how to improve our well-being inside buildings.

For conceiving such an improved concept, we sampled different built environments, characterized by an increasing level of microbial control, (public buildings, public and private houses, intensive care units, cleanroom facilities) in a large scale, to evaluate associated structural and functional changes of the microbiome. Genome centric shotgun metagenomics were applied to investigate functional capabilities of similar species from different built environments. All data was supported by gene centric analysis, 16S rRNA gene amplicons, qPCR, and detailed metadata.

Microbial maintenance indoors changed environmental conditions for microbes: The increased level of microbial control was negatively correlated with the diversity of its biome and showed less complex microbial interactions and functional networks. On the contrary, human associated particles and microbes such as *Propionibacterium acnes*, *Staphylococcus aureus*, *Acinetobacter baumannii* and *Stenotrophomonas maltophilia*, showed higher relative abundances in more controlled built environments. However, naturally uncontrolled built environments were mainly influenced by microbes from the outdoor environment (soil: *Exiguobacterium sibiricum*, air: *Janibacter hoylei*, plants: *Kocuria palustris*) and foods (dairy products: *Lactococcus garvieae*, meat: *Macrococcus caseolyticus*, *Streptococcus parauberis*, and fermented foods: *Weissella confusa*). Both opposing environments could not only be characterized by discriminant profiles of its species (Archaea, viruses or arthropods inside confined built environments), but also by numerous functional features. According to this analysis, uncontrolled built environments were populated by gram-positive bacteria with functions associated to carbohydrate and amino acid metabolism, while controlled built environments were characterized by gram-negative bacteria with many functions associated to virulence, disease and defense. This trend was also visible on the level of binned genomes. Hence, genomes closely related to *Acinetobacter* and derived from all sampled built environments revealed a higher proportion of genes coding for virulence, disease, resistance or defense in microbial controlled built environments. Finally, correlations of the dataset on structural and functional level with measured environmental parameters indicated

the type of the built environment as a main driver for microbial diversity profiles, beside biogeography and different building materials of sampled surfaces.

This study indicates which conditions enforce a microbiome to develop adverse functional capabilities and might serve as a model for attempts to better design and control (biotechnologically) microbiomes indoors in the future for human health.

Introduction

The man-made built environment is increasingly covering the face of planet Earth (Martin *et al.*, 2015). However, this environment not only provides living space for humans, but also uninvited to diverse microbial communities. These microbial communities followed mankind through different stages of urbanization and evolution in the built environment from simple shelters of the outdoor environment to multi-functional modern buildings (Ruiz-Calderon *et al.*, 2016). Nowadays, buildings are the main environment where people spend their lives, share microbes, and many diseases of civilization may have its origin. Hence, research targeting the microbiology of built environments moved into the spotlight for many labs. Studying the microbiology of the built environment focused very much on different drivers like architecture (Kembel *et al.*, 2012), ventilation (Meadow *et al.*, 2014), room usage (Flores *et al.*, 2011, 2013), occupancy (Meadow *et al.*, 2015), the interaction of its residents with the indoor environment (Lax *et al.*, 2014), microbial sources in general (Adams *et al.*, 2015; Mahnert, Moissl-Eichinger, *et al.*, 2015) and impacts on the microbial structure indoors (Dunn *et al.*, 2013; Kelley and Gilbert, 2013).

Microbial profiles were also shown to be very much affected by maintenance and microbial confinement (Moissl-Eichinger *et al.*, 2015; Mahnert, Vaishampayan, *et al.*, 2015; Oberauner *et al.*, 2013). However, an unselective removal and killing of all microbes in the built environment could have adverse health effects (Arnold, 2014; Blaser, 2011; Blaser and Falkow, 2009), since potent immune development may be dependent on microbial exposure especially during infant development (Eder *et al.*, 2006; Vatanen *et al.*, 2016; Gensollen *et al.*, 2016; Hofer, 2016; Rook, 2009; Warner, 2003).

Hence, we were interested in effects of microbial confinement and control in the built environment and how these actions not only affect microbial community structure, but also much more the functional capabilities of the microbiome. For this purpose, we defined several model built environments differing in their grade of microbial confinement and restrictions to access these environments. On the one hand, we investigated different naturally uncontrolled buildings and houses with a high impact from the surrounding outdoor environment including plants in a rural setting. On the other hand, we sampled controlled built environments with an increasing level of microbial confinement from intensive care units (ICUs) to spacecraft assembly cleanroom facilities in urban areas. All samples were supported by a rich collection of

environmental metadata for further correlations between the microbiome and environmental parameters.

Reports about the functional capabilities of microbiomes in the built environment are still scarce. However, a recent study investigated not only microbial, but also functional diversity of microbes inside a spacecraft assembly cleanroom facility (Weinmaier *et al.*, 2015). This study bypassed the problem of overall low biomass inside controlled built environments by amplifying DNA for shotgun metagenomics via MDA (multi displacement amplification). As this method could introduce similar biases like PCR, we focused on large scale samplings of big indoor spaces with adapted sampling methods instead. This approach allowed the acquisition of enough biomass for direct shotgun metagenomics and a state-of-the-art genome centric analysis (Turaev and Rattei, 2016). Shotgun metagenomics analysis was additionally supported by a 16S amplicon analysis and qPCR assays.

Hence, this comprehensive study design allowed us to reveal effects of microbial maintenance on the functional capabilities of the microbiome in the built environment. In addition, we could develop a model for these human driven processes and improved our assessment of how we could biotechnologically design microbiomes in the built environment not only on a structural, but also at a functional level for the benefit of future human health in the built environment.

Materials & Methods

Environmental parameters and study design

A variety of indoor environments differing in respective levels of microbial control, maintenance and access were sampled during the same season of the year (spring, vegetation period of the year), to reveal possible effects of microbial control on the microbiome in built environments. Public buildings as well as public and private houses located in the rural area of a wildlife park (in close touch with nature in Eekholt, Germany) represented the model for a totally uncontrolled built environment (Supplementary Fig. S1A). The intensive care unit (ICU, Department of Internal Medicine, University hospital, Graz, Austria, Supplementary Fig. S1B) in full operation and in a suburban area, act as a model for moderate restricted built environments with special attributes of its occupants (Oberauner *et al.*, 2013). Finally, a highly controlled indoor environment was represented by a cleanroom facility with its adjacent gowning area used for spacecraft assembly by the European Space Agency (ESA) in the urban area of Turin, Italy (Supplementary Fig. S1C). Samples throughout these different built environments were obtained from floors to allow a high grade of comparability and sufficient biomass for the applied methods. In addition, samples from ICU floor surfaces were compared with samples from other ICU locations as well, using a targeted approach of the 16S rRNA gene.

All these indoor environments feature different environmental parameters summarized in Table 1, which are suspected to contribute to certain magnitudes to the structure and function of its microbiome. In particular the following environmental influences should be considered for each sampled category of a built environment:

The natural uncontrolled indoor environment of public and private buildings and houses in a rural area were exposed to the highest degree to the surrounding outdoor environment. A variety of discrete landscapes with respectively associated ecosystems surrounded these structures – including bogs, wetlands, coniferous and deciduous wooded forests with a winding creek in grasslands and pasture including > 700 animals in ~100 different species of insects, fish, reptiles, birds and mammals (www.wildpark-eekholt.de).

The public buildings were located in all these different ecosystems. During the business hours of the wildlife park (from 9 am to 6 pm) these buildings were wide apart for a microbial input via the outdoor air (pollen, seeds, water droplets and other biotic and abiotic particles and

compounds) and various animals (insects, birds, mammals, humans and their pets) with brought along particles associated to soil, foods (snacks), and personal items (e.g. buggies etc.). The rooms of these structures were window ventilated or simply wide apart. Floor surface materials comprised concrete, tiles, polymers and wood. In contrast to the other sampled indoor environments the floors were cleaned only mechanically with a broom and people interacted only marginally with the floor environment, since they primarily observed wildlife, studied boards about the presented ecosystem or simply seek for shelter due to unfavorable weather conditions.

The public houses were so-called 'A-frame houses' surrounded by lawns, conifers and deciduous trees adjoining to broad fields of farmland. The houses comprised a recreation room (wooden floor), a kitchen and restrooms (tiled floors). Especially the floor surfaces of the kitchen and the restrooms were treated regularly with natural soaps and were also affected (food preparation, conducting daily hygiene) in a different way compared to the previous category of public buildings. The public houses were used by school classes, who occupied the rooms for a week to receive environmental education. Houses were closed during absence of occupants and window ventilated in the presence of pupils or the cleaning staff.

While a public house is frequented by numerous temporary residents, the private house in contrast is usually inhabited by a family or a reduced number of residents. Likewise, daily behavior and interaction with surfaces is highly personalized. Hence, compared to public houses, this personalized maintenance represents a first step of confinement of an indoor environment. The sampled private house was an old farmyard encircled by a garden with fruit trees and conifers of the adjacent forest. Sampled rooms comprised a kitchen (floor with polymer tiles), a barn mainly used for dining with guests (stone floor) and a conference and locker room (tiled floor) in an adjacent building used as a bird foster station. The house was window ventilated and occupied by the co-founder of the wildlife park - an elderly women and her dog. The resident was supported by a household help and regularly visited by employees of the wildlife park. Floors were cleaned with all-purpose cleaners, the dog received its food in a bowl in the kitchen and the barn was inhabited by swallows during the summertime. Due to the high frequentation of occupants it was uncommon to change shoes before entering the private house (in contrast to many other private households). The same behavior was true for the public built environments described above.

On the contrary to these uncontrolled built environments, the investigated ICU was located in a suburban area. Compared to public buildings, public and private houses, indoor environments

of hospitals are severely different in many aspects: First of all beside staff the majority of people are suffering from severe infections and other health problems. Therefore these patients might deliver specific microbes like opportunistic pathogens to their surroundings or acquire problematic germs from it. In this ICU critically affected patients suffering from all subspecialties of internal medicine and neurologic defects were treated in 15 beds including an isolation unit for severe immunocompromised patients. All rooms of the ICU were mechanical ventilated by an air conditioning system. Access to the ICU was restricted and controlled by the medical staff. Special garment regulations were applicable in dependence on the grade of interaction with patients or medical devices. A comprehensive maintenance, cleaning and disinfection plan especially characterizes this indoor environment: Waste was removed three times a day and waste containers were disinfected with a surface disinfection cleaner. In the same manner doorknobs and tiles in working height were cleaned twice per day. All-purpose cleaners were used to treat free surfaces and furniture (shelfs, chairs, tables, conduits, window boards, radiators, elevators), a surface disinfectant was used for dirty laundry buckets, handrails, cleaning cars, surface cleaning devices and sanitary glass cleaners and surface disinfectants were used at the bath place for showers and sinks once a day. All other furniture, objects or room installations were cleaned weekly, monthly, per quarterly period, semiannual or annually. Beside restrooms and shower heads all floors were cleaned twice a day or even more often if visible contaminants (blood, feces or other secreted liquids) were observed. If known hospital germs (e.g. *Clostridium difficile*) were detected additional hygiene protocols became effective up to individual isolation of patients. A detailed list of all cleaning products used for disinfecting hands, surfaces, instruments or antisepsis of skin and mucosa are listed in Table S1 A and B beside other agents to treat known resistant hospital germs and viruses.

An additional level of microbial control is realized in cleanrooms during spacecraft assembly, especially if a celestial body with relevance to extraterrestrial life detection is targeted. According to planetary protection regulations (Nicholson *et al.*, 2009) these spacecraft are not only assembled in cleanrooms, but are also subject to regular microbial monitoring, cleaning and removal of surface associated microbes (bioburden control and countermeasures) (Rummel *et al.*, 2002). Most microbes are intensively reduced not only by the low water and nutrient availability, but especially through intensive HEPA air filtration and the reduction of air-borne particles through air recirculation. Hence, cleanrooms are categorized into different cleanroom classes by the amount and size of particles. Moreover, type and quality of gaseous substances, temperature,

humidity, electromagnetics, electrostatics etc. can be controlled. Low emitting surfaces and materials (e.g. floors, doors, door handles, tables, shelves, trolleys) are mopped with sterile cloths to remove dust, before surfaces are cleaned several times a day with alcohols (e.g. 70% isopropanol), detergents based on hydrogen peroxide (e.g. Klercide-CR), high alkaline cleaning reagents (e.g. Jaminal Plus or Kleenol 30) and sometimes even with vapor-phase hydrogen peroxide or cold plasmas to reduce the bioburden of spacecraft. However, due to the complexity of working processes during spacecraft assembly, engineers still have to interact with spacecraft components and are therefore a main source of microbial dispersal. As a counter measurement strict gowning protocols with special cleanroom garment have to be followed in repetitive steps and sluices and air showers define transitions through different cleanroom classes and rooms. Finally strict protocols (e.g. slow body movements) regulate the interaction of engineers and staff with the cleanroom environment, spacecraft components and the assembled spacecraft itself to minimize the dispersal of human associated microbes into the cleanroom.

All floors of indoor environments with different microbial restrictions were investigated in respect of the main categories: 'uncontrolled' (public buildings, public and private houses), 'moderate controlled' (ICU), and 'highly controlled' (cleanroom facility) with comparative shotgun metagenomics to resolve microbial profiles and their associated functions. A higher resolution of individual samples was further achieved by 16S rRNA gene amplicons and predictions of its potential functions via PICRUSt (Langille *et al.*, 2013).

Sampling procedures

Floor samples in a large scale (defined by the size of a room) were collected to obtain high amounts of biomass (even from low-biomass environments like cleanrooms). For this approach sterile (autoclaved) and DNA-free (dry heat treatment) Alpha Wipes® (TX1009, VWR International GmbH, Vienna, Austria) were mounted in several layers separated by sterile, DNA-free foliage on a big swab (Swiffer® Sweeper® Floor Mop Starter Kit, Procter & Gamble Austria GmbH, Vienna, Austria) under a biohood. If necessary, wipes were remoistened with PCR-grade water that was sprayed directly on the surface with an atomizer. All instruments were chemically sterilized in several steps (all-purpose cleaner, Denkmit, dm-drogerie markt GmbH + Co. KG, Karlsruhe, Germany; 70% (w/v) ethanol, Carl Roth GmbH & Co KG, Karlsruhe, Germany and Bacillol® plus, Bode Chemie GmbH, Hamburg, Germany). The remaining DNA was removed

with chlorine bleach (DNA away, Molecular Bio Products, Inc. San Diego, CA, USA) and UV light (254 and 366 nm, Kurt Migge GmbH, Heidelberg, Germany). Samples were collected in a repetitive way, always starting from the cleaner areas in each indoor environment (especially cleanrooms) to minimize the transfer of contaminants and by one person only to guarantee a consistent sweeping pattern (horizontally, vertically and diagonal sweeping motion) as well as a consistent uptake of particles and microbes. Samples were stored on blue ice and processed at the laboratory within 12 h after each sampling event. Samples from the ICU were already obtained and processed in a previous study (Oberauner *et al.*, 2013).

Sample processing, PMA treatment and DNA extraction

Samples were processed, concentrated, treated with PMA and DNA extracted as described before (Mahnert *et al.*, 2015). PMA treatment of samples from high-biomass environments were performed as an additional control for potential DNA contaminants in used reagents and on sampling equipment. In addition, PMA treatment served as a proxy to evaluate proportions of intact microbial cells and validate drawn conclusions on viable microbial cells in the dataset. The DNA extraction method with the XS buffer was suitable for low-biomass environments, however for samples from high-biomass environments additional cleaning procedures were performed with the Geneclean® Turbo Kit (MP Biomedicals, Heidelberg, Germany) according to manufacturer instructions.

Quantitative measures

The bacterial abundance was investigated for most samples as described in (Mahnert *et al.*, 2015) with the primer pair 515f – 927r (10µM each) in 40 cycles of a qPCR run (denaturation at 95°C for 20 sec, annealing at 54°C for 15 sec and elongation at 72°C for 30 sec) on a Rotor-Gene™ 6000 real – time rotary analyzer (Corbett Research, Sydney, Australia). A melt curve from 72°C to 95°C served as a quality control for the amplified products. One qPCR reaction mix was constituted as follows: 1.06 µl PCR grade water, 3.5 µl KAPA Plant PCR buffer (KAPA3G Plant PCR Kit, Peqlab, VWR International GmbH, Erlangen, Germany), 0.42 µl forward and reverse primers, 0.056 µl of KAPA3G Plant DNA-polymerase (2.5 u/µl), 0.78 µl of SYBR® Green (4x concentrate, Invitrogen™, Eugene, OR, USA), and 0.8 µl of the extracted DNA

template. qPCR runs with a mean reaction efficiency of 0.84 and mean standard curve R^2 values of 0.99 (16S rRNA gene product of *Bacillus subtilus* B2G) were evaluated in triplicate and counts in negative controls were subtracted from all other samples in their respective qPCR runs.

Shotgun metagenomics

Total extracted DNA of all samples pooled into the categories public buildings, public houses, private houses, ICU, cleanroom and gowning area with a mean DNA amount of ~ 10 µg and a mean DNA concentration of 149 ng/µl were send to Eurofins Genomics GmbH (Ebersberg, Germany) for sequencing on an Illumina HiSeq 2500 instrument with 2 x 150 bp paired-end in the rapid run mode. After quality control, 9 shotgun libraries were prepared by fragmentation and end repair of DNA with an insert size of ~300 bp. Libraries were individual indexed for multiplexed sequencing after PCR amplification, library purification and final quality control of all libraries. Libraries were purified with gel extraction, quantified, mixed in one pool and sequenced.

16S rRNA gene amplicons

Amplicons targeting the 16S rRNA gene were generated with the barcoded primer pair 515f – 806r (Klindworth *et al.*, 2013; Caporaso *et al.*, 2010). Four individual PCR reactions à 50 µl (17.6 µl PCR grade water, 25 µl KAPA Plant PCR buffer, 0.4 µl KAPA3G Plant DNA-polymerase (2.5 u/µl), 3 µl forward and reverse barcoded primers and 1 µl extracted DNA template) were pooled after successful amplification (40 cycles of 95°C for 30 sec denaturation, 60°C for 15 sec annealing and elongation at 72°C for 12 sec) on a TECHNE TC-PLUS gradient thermocycler (Bibby Scientific Ltd, Stone, UK) and validation via gel electrophoresis. Pooled PCR products were cleaned with the Wizard SV Gel and PCR Clean-Up System kit (Promega, Madison, WI, USA) and measured on the NanoDrop instrument (Thermo Scientific, Wilmington, DE, USA). Equimolar concentrations of PCR amplicons were pooled and send for sequencing at Eurofins Genomics GmbH, Ebersberg, Germany. The pool was additionally purified via gel extraction and quantified before the sequencing library was prepared with ligation of adaptors, PCR amplification according to PCR product insert size and a final library purification and quality control. Sequencing of amplicon samples was performed on an Illumina MiSeq

instrument with v3 chemistry with the 2 x 300 bp paired-end read module. Amplicon sequences of the ICU were generated and sequenced as described in (Oberauner *et al.*, 2013).

Controls

Beside the actual samples negative controls were processed at each experimental step. Counts in negative controls were subtracted from the qPCR data. PMA treatment served as an additional quality control for used reagents, equipment and overall observations. Extraction controls and field blanks (samples of the background environment without contact to the floor surface) were processed in parallel. Sequences of these control samples were removed from the dataset during the bioinformatical analysis.

Bioinformatics

Shotgun metagenomics:

After quality control of raw reads, sequencing adapters were removed from sequences and quality filtered according to phred score (> q35) as well as length filtered (min. 50 bp) by trimming from the 3' prime site. The whole analysis was conducted in a genome centric approach focusing on assembly based data (contigs, scaffolds and bins). However, gene centric analysis based on single reads served as a quality control for assembly-related artifacts throughout the analysis. These single reads were assigned using the blastx search algorithm against a marker database of Archaea, Bacteria and Eukaryota as well as against the NCBI non-redundant database (release of September 2015). Annotations of all single reads were determined and analyzed with MEGAN (Huson *et al.*, 2007). For the genome centric approach quality sequences were assembled with Ray Meta and a max. kmer-length of 32 (Boisvert *et al.*, 2012). Assemblies were filtered according to the following parameters: min_length 1500, min_coverage 5, and readlength 150. A summary of all filtered assemblies is provided in Table S2. Afterwards, filtered contigs were taxonomically classified with AMPHORA2 (Wu and Scott, 2012) using the database of markers described above. For the visualization in Krona charts, the coverage ratios of respective contigs were considered for showing relative abundances. Contigs were further binned through a genome centric multiple coverage based approach with CONCOCT (Alneberg *et al.*, 2014) and MaxBin (Wu *et al.*, 2014). Binning quality of contigs was validated with CheckM (Parks *et al.*,

2015) and bins were visualized with VizBin (Laczny *et al.*, 2015). Contigs of each bin were re-annotated with AMPHORA2, and compared with publicly available genomes using RAST (Overbeek *et al.*, 2014) and MaGe (Vallenet *et al.*, 2006) to reveal ecological relevant functional subsystems. Predicted functional classifications of protein-coding genes were analyzed by annotation and comparative genomics in IMG (Markowitz, Chen, Palaniappan, *et al.*, 2014; Markowitz, Chen, Chu, *et al.*, 2014) with GO terms (Gene Ontology Consortium, 2000), KEGG (Kanehisa *et al.*, 2012) and SEED classifications (Overbeek *et al.*, 2014). Shotgun reads are accessible in MG-RAST (http://metagenomics.anl.gov/) under projects 9258 and 10962, binned contigs and scaffolds are deposited in IMG/M (https://img.jgi.doe.gov/) through the Genome Online Database GOLD (https://gold.jgi.doe.gov/) (XXX).

16S rRNA gene amplicons:

Sequences of 16S rRNA gene amplicons of all indoor environments were analyzed in QIIME 1.9. (Caporaso *et al.*, 2010) according to the Illumina Overview Tutorial. Forward and reverse reads were joined with a minimum overlap of 100 bp and a maximum allowed difference of 3%. Barcodes were extracted for demultiplexing and quality filtering of reads with default parameters. Reads in controls were removed by blast (100% alignment cutoff). 454 reads of ICU samples were denoised with mothur (Schloss *et al.*, 2009), demultiplexed and quality filtered before they were concatenated with the filtered Illumina reads. All sequences were then trimmed to the same 16S rRNA gene regions and lengths (FASTX-toolkit by Assaf Gordon, accessed on Galaxy, galaxyproject.org). Reads were additional quality filtered to remove primer sequences before chimeric sequences were removed with usearch (Edgar, 2010) giving the Greengenes 13_8 release as a reference. OTUs were picked with usearch to the same reference and every sequence not present was clustered denovo at 97% similarity level and a phylogenetic tree was calculated for phylogenetic based metrics and measures. The OTU table was filtered for singletons, doubletons, assigned reads to chloroplasts or mitochondria and sorted for following read normalizations in alpha and beta diversity analysis and statistics. Since jackknife supported bootstrapped trees (Supplementary Fig. S2) showed higher confidence for weighted than unweighted measures, weighted metrics were preferred throughout the analysis. OTUs present in the reference were examined for their functional potential via PICRUSt (Langille *et al.*, 2013). OTU networks were based on the assigned taxonomy, calculated in QIIME and visualized in Cytoscape (Shannon *et al.*, 2003) as described earlier (Moissl-Eichinger *et al.*, 2015). Raw 16S

rRNA gene amplicon reads were deposited in the European Nucleotide Archive (www.ebi.ac.uk) under project XXX (XXX).

Statistics

Additional statistics were conducted in QIIME (calling respective R-scripts) or directly in R using the vegan package. Statistical tests included a comparison of categories, distances, distance matrices, core microbiomes and core functions, taxa summaries, co-occurrence patterns, correlations of metadata and a BIO-ENV test. For nonparametric tests like multi response permutation procedures (MRPP), adonis, ANOSIM, distance comparison boxplots, Mantel correlograms and Mantel tests, statistical significance was determined through 999 permutations. Distance comparison boxplots were calculated using a two-sided Student's two-sample t-test. Resulting P-values were Bonferroni-corrected. PCoA plots were based on weighted unifrac metrics. The NMDS were calculated from a Bray-Curtis distance matrix. Vectors of environmental variables shown in the NMDS were calculated with the BIO-ENV function based on Euclidean distances in R as were calculated ellipses per sample group.

Linear discriminant analysis of the effect size (LEfSe) (Segata *et al.*, 2011) and microbiome: picking interesting taxonomic abundance analysis (microPITA) (http://huttenhower.sph.harvard.edu/micropita) were performed on Galaxy modules provided by the Huttenhower lab. Both tools were executed with default settings using an all-against-all strategy for the multi-class analysis for 16S rRNA gene amplicon data sets and a one-against-all strategy for the PICRUSt predicted functions throughout the LEfSe analysis.

Results

3.0 - 6.7 x 10^7 sequences per sample could be obtained via shotgun Illumina HiSeq sequencing. After filtering, a range of 7 x 10^6 to 2.5 x 10^7 quality sequences (phred score ≥ q36) could be retained for following assemblies and binning attempts (Supplementary Table S3). Assemblies on Ray Meta resulted in a satisfying amount of small (≥ 100 nt) and large (≥ 500 nt) contigs and scaffolds (range of N50 values: 142 to 2510, Supplementary Table S2). From these contigs and scaffolds 304 bins (125 high quality bins; 8 to 20 bins per sample) could be generated. Most bins were obtained from the private house, while only a few genomes could be binned from the ICU dataset (Table 1). In general, most markers (1389) and marker sets (369) could be binned to 39 genomes assigned to the genus *Pseudomonas* with a completeness of 93.47%, contamination of 2.51%, and heterogeneity of strains of 26.67%.

Supportive 16S rRNA gene amplicon analysis for higher sample resolutions of respective built environments resulted in 225 to 37,831 sequences per sample from a total of 837,216 quality sequences and 10,814 assigned OTUs (Supplementary Table S4).

The structure of the biome in the built environment was shaped by environmental differences.

All sampled built environments could be characterized by distinct environmental features such as the geographic location, microclimate, architecture, room maintenance and usage. Whereas samples from uncontrolled environments were located at low elevations in northern Germany, samples from controlled built environments originated from higher altitudes in south-east Austria and north-west Italy. Concerning respective microclimates, uncontrolled environments of public buildings and public as well as private houses were specified by higher relative air humidity and lower temperatures, compared to controlled environments of the ICU, and the cleanroom facility with its gowning areas. The ICU highlighted most opposed climatic conditions: with the highest room temperature (24°C) and the lowest relative air humidity (32%). Further discriminating attributes were represented by architectural features of sampled built environments. Here, cleanrooms represented very large indoor room surfaces compared to small spaced infrastructure in the case of gowning areas, rooms in the ICU, public buildings and public and private houses. The smallest room volumes were represented in private houses. Likewise, more controlled built environments harbored only a few defined synthetic materials on floors, compared to a higher

variety of floor materials including materials of natural sources like wood and stone in uncontrolled built environments. Moreover, occupancy and intensity of cleaning were obviously different between public buildings, public and private houses, the ICU and the cleanroom facility with its gowning areas. Hence, a higher grade of environmental confinement resulted in more elaborative maintenance and cleaning procedures, but a lower grade of occupancy.

Environmental differences were also mirrored in the biome structure of these built environments: PCoA ordinations and UPGMA (Unweighted Pair Group Method with Arithmetic Mean) trees showed a higher taxonomical and functional similarity of samples from public buildings with public houses; samples from controlled built environments (ICU and cleanroom facility) towards samples obtained from private houses (Fig. 1 A and B). Even on superkingdom level an obvious pattern of the entire biome structure (based on single reads; blastx vs. NCBInr; excluding unassigned reads) was visible (Fig. 2A). Whereas the number of reads assignable to Bacteria was dominant in samples obtained from public buildings, public houses and private houses (~ 99%); this proportion decreased significantly (T-Test, P-value = 0.04) towards confined built environments (ISO 8 cleanroom ~ 69% and its gowning area ~ 85%; ICU ~ 55%). In contrast, these confined built environments were characterized by a significantly (T-Test, P-value = 0.04) higher proportion of Eukaryota (ISO 8 cleanroom ~ 31% and its gowning area ~ 15%; ICU ~ 44%;) compared to the uncontrolled built environments (~ 1%). A similar pattern could be observed for Archaea, although not significant, with higher counts (~ 4 times) in controlled than uncontrolled built environments. Patterns of Viruses were less apparent between the controlled and uncontrolled built environments, but showed highest relative abundances in the ICU and in the environment of public houses. Hence, this data revealed higher similarities of different uncontrolled built environments (public buildings, public houses and private houses) than for different confined built environments (ICU, cleanroom facility with gowning area).

Diversity gets reduced towards private and more controlled built environments.

Patterns of the biome structure found consonance into diversity estimates of natural uncontrolled and more confined built environments (Supplementary Table S5 and Fig. S3 A and B). Whereas public buildings and houses revealed a significantly higher diversity (T-Test P-value 0.007) of ~ 8.7 H' (Shannon-Weaver index), diversity decreased in more confined built environments (cleanroom and gowning area ~ 7.6 H', ICU 6.3 H') and towards private houses ~

5 H' in the presence of a comparable range of total bacterial abundance (~ 10^6 – 10^7 16S rRNA gene copies per m^2; with a higher variability of the viable bacterial abundance of (~ 10^3 – 10^7 16S rRNA gene copies per m^2). On the contrary, functional diversity (based on KEGG and SEED annotations) was much more evenly distributed and was highest in the ICU (12.1 according to KEGG and 11.1 according to SEED annotations) and again lowest for private houses (11.3 according to KEGG and 10.8 according to SEED annotations).

Microbial maintenance of an indoor environment had obvious effects on microbial profiles

Clear differences of the microbiome structure were continued into higher taxonomic levels. Hence, on phylum level (Fig. 2B) public buildings and public houses were characterized by a high relative abundance of *Actinobacteria* (up to 50%) and *Proteobacteria* (~ 21%). However, this pattern changed in the private house and if sequences of intact cells were targeted via PMA pretreatment towards a predominance of *Firmicutes* (up to 55%). For microbial controlled built environments (ICU, ISO 8 cleanroom and adjoining gowning area) the prevalence of bacterial phyla was reduced and proportions of cellular organisms and not assignable sequences increased by a maximum of 62% especially in the environment of the cleanroom.

Taxonomic assignment of contigs with AMPHORA2 (correlated with the respective coverage of each contig) and single reads identified obvious discriminative patterns for different sampled built environment categories even on highest taxonomic levels. Hence, as shown in Fig 2C, public buildings showed a high relative abundance of *Nocardioides* sp. CF8, *Arthrobacter* sp. PAO19, *Knoellia aerolata*, *Janibacter* sp. HTCC2649 and *Janibacter hoylei*. Public houses were dominated by signatures of *Exiguobacterium sibiricum*, *Microlunatus phosphovorus*, and *Kocuria palustris*. On the contrary, in private houses most sequences could be assigned to *Streptococcus parauberis*, *Lactococcus garvieae*, *Weissella confuse*, *Leuconostoc mesenteroides*, *Staphylococcus vitulinus*, and *Macrococcus caseolyticus*. Likewise, confined built environments revealed their own distinct microbiome dominated by human sequences and *Propionibacterium acnes*, *Staphylococcus aureus* and *Pseudomonas putida* (especially in ICU environments), and dominant patterns of *Stenotrophomonas maltophilia*, *Acinetobacter johnsonii* and *A. baumannii* in samples of the cleanroom facility.

Notable, the following bacterial taxa (e.g. *Pseudomonas*, *Porphyromonas*, *Propionibacterium*, *Prochlorococcus*), viral sequences (e.g. human herpes and papillomavirus) and assignments to

arthropods (e.g. mites like *Trombidiformes* and *Prostigmata*) and insects (e.g. lices such as *Liposcelis bostrychophila* and cockroaches like *Blattella germanica*) could be identified as a significant discriminative feature for controlled built environments (Fig. 2D) via LEfSe (linear discriminant analysis of the effect size) analysis.

Co-occurrence networks (Fig. 3 A and B) revealed associations of several bacteria with humans (*Propionibacterium acnes*, *Acinetobacter baumannii* and *A. johnsonii*), other mammals like pets (*Leuconostoc* sp. DORA_2 and *Bordetella bronchiseptica*), archaea (uncultured marine thaumarchaeote KM3) and other mammals. Bacteria found in kitchens formed co-occurrence patterns probably due to food processing (*Lactococcus garvieae* and *Macrococcus caseolyticus*), food digestion and actions on restrooms of private and public buildings (*Enterococcus faecalis*, *Staphylococcus vitulinus*, *Streptococcus parauberis*, *Leuconostoc mesenteroides*, *Lactococcus lactis*, *Weisella confusa* and *W. cibaria* and *Enterococcus faecium*, *Janibacter hoylei*, *Knoellia aerolata*, *Arthrobacter* sp.). Moreover, host associated species of plants and humans (*Agrobacterium tumefaciens*, *Pseudomonas putida*, *Stenotrophomonas maltophilia*, *Massilia timonae*) formed co-occurrence networks as did halophilic and thermophilic species (*Nocardioides halotolerans* and *Pyrinomonas methylaliphatogenes*) from soil. In general, higher co-occurrences were present between samples from uncontrolled than controlled built environments.

For a deeper analysis and investigations on higher resolutions per sample, 16S rRNA gene amplicons were generated. MicroPITA (microbiome: Picking Interesting Taxonomic Abundance) analysis was used to identify samples with the maximum diversity, representative dissimilarity, most dissimilar, or discriminant features. Interestingly, according to this analysis samples from controlled built environments contained most often the maxima for representative and highest dissimilarity, while samples from medical devices, workplaces and the private houses represented most often the minima in these categories. On the other hand, samples from uncontrolled built environments were more often representative for distinct and discriminant features. In contrast ICU samples represented most often minima in these categories.

This higher resolution of sampled built environments showed that many microbes where present not only in uncontrolled environments of public buildings and public as well as private houses, but even in controlled areas of the cleanroom facility as highlighted by a core microbiome OTU network (Fig. 4) and a heatmap on phylum level (Supplementary Fig. S4).

Hence, *Acinetobacter*, *Bacillus*, *Exiguobacterium*, *Macrococcus caseolyticus*, *Lactococcus garvieae*, *Sphingomonas*, *Arthrobacter*, *Kaistobacter*, *Kocuria palustris* and *Nitrososphaera,* to name a few of the most relative abundant sequences of bacteria and archaea, were shared in the core microbiome of these two opposed built environments. Some of these taxa were even common in the environment of the ICU e.g. *Acinetobacter*, *Sphingomonas*, *Methylobacterium*, *Bacillus cereus*, *Agrobacterium*, *Staphylococcus*, *Streptococcus*, *Pseudomonas*, and *Micrococcus*. Notable, *Propionibacterium acnes* was present as an exclusive OTU of its core microbiome in ICU environments. On the other hand, *Acinetobacter* showed a very high relative abundance in public buildings beside *Arthrobacter*, or *Macrococcus* (wooden floor), *Exiguobacterium* (polymer floor) and *Kaistobacter* (tiled floor). However, for environments that were influenced by food preparation or consumption, floor surfaces revealed a high relative abundance of *Lactococcus* (in kitchens, dining halls and recreation rooms). Hence, public and private houses that included these environments and even restroom surfaces featured a more distinct microbial composition. Here, *Acinetobacter* were relatively reduced towards *Exiguobacterium*, *Erwinia*, *Kocuria*, *Enhydrobacter*, and *Bacillus*. In the private house *Staphylococcus* increased in terms of relative abundances (beside other common genera like *Macrococcus*, *Lactococcus* and *Arthrobacter*). Whereas ICU floors harbored a high relative abundance of *Staphylococcus* and *Acinetobacter*, sampled medical devices could be characterized by a broad set of sequences assigned to *Propionibacterium*, *Legionella*, *Streptococcus*, *Burkholderia*, *Pseudomonas*, *Novosphingobium*, and *Nitrospira*. Workplaces of the medical staff in the ICU harbored additionally species of *Curvibacter*, *Bacillus*, *Paenibacillus* and *Janthinobacterium*. In contrast, samples from the gowning area included sequences that could be annotated to *Deinococcus*, *Corynebacterium*, and *Planctomyces* beside a general abundance of *Acinetobacter*. All three sampled cleanrooms of ISO 7 and ISO 8 classification revealed a divergent microbial composition. Hence, whereas the ISO 7 cleanroom that harbored first parts of the actual assembled spacecraft contained many sequences of *Acinetobacter*, *Arthrobacter* and *Micrococcaceae*, the adjoining cleanroom next to the exit into the ISO 8 area and equipped with a material sluice revealed signatures of *Lactococcus* and surprisingly a relatively high abundance (7.6%) of the Thaumarchaeote *Nitrososphaera* (additionally classified to the Crenarchaeal soil group with the SILVA 119 release). Finally, the microbial composition of the ISO 8 cleanroom was dominated by *Acinetobacter*, *Xenococcaceae* and *Xanthomonadaceae*. Discriminant features on taxon level were further identified via LEfSe analysis (Fig. 5A). Hence, *Archaea* were

identified as a discriminant feature for controlled built environments, *Sphingomonadaceae*, *Paracoccus* and *Rhodobacterales* for moderate controlled built environments of the ICU, and finally beside unassigned sequences, *Deinococci*, *Thermi*, *Isosphaeraceae*, *Methylibium*, *Phycicoccus*, *Actinomycetospora*, *Microbacteriaceae*, *Pseudonocardia* and *Rubrivivax* for sampled uncontrolled built environments.

Similar patterns of environmental confinement could be observed at the level of alpha diversity. Here, compared by its median (Fig. 5B), public buildings showed the highest diversity (> 8 H', Shannon-Weaver index), followed by public houses, sampled cleanrooms and their gowning areas (> 7.5 H') and the private house (> 6 H'). On the contrary microbial diversity of the ICU was low with the highest diversity on floors (4 H'), then workplaces (> 3.5 H') and lowest on medical devices (> 3 H'). A more detailed look revealed further differences of samples from restroom surfaces (> 7.5 H'), kitchens (> 7 H'), the barn (7 H') and locker rooms (6 H') of uncontrolled built environments.

Even on the level of beta diversity, based on either weighted or unweighted unifrac metrics, samples from the ICU clustered more separately from private and public buildings and houses, than samples obtained from cleanrooms and their gowning areas along reasonable explained distances of 35.61% with PC1 and 20.05% with PC2 (Fig 5C). Further data analysis was focused on weighted unifrac metrics, since jackknife UPGMA boostrapped trees based on weighted unifrac measures showed higher trust indices of respective branches in the dendrograms (Supplementary Fig. S2). Overall samples from floors clustered closer than those obtained from workplaces or medical devices. Likewise, samples from the private houses were more similar to samples from ICU floors. A multi response permutation procedure (MRPP, see Table S6A for additional statistics) based on the weighted unifrac metric showed significantly distinct microbial community structures between controlled, moderate controlled and uncontrolled built environments (observed delta 0.42, expected delta 0.5; with a fairly high chance corrected within-group agreement of 0.1618), that were higher than the separation of microbial communities in terms of the sampling location (floors, workplaces, medical devices; chance corrected within-group agreement of 0.1583). In addition, adonis tests (R^2) and analysis of similarities (ANOSIM) indicated different types of built environments as a main driver for observed dissimilarities of weighted unifrac metrics in the structure of the microbiome. Hence, the highest proportion of distance (51%) as well as the strongest grouping per individual (R = 0.61) could be explained by the type of the built environment compared to sampled surface locations (30%; R = 0.60) or

surface materials (42%; R = 0.38). A series of two-sided two sample Student's t-tests indicated higher distances between controlled vs. moderate controlled (distance = 0.6) than controlled vs. uncontrolled (distance = 0.4) built environments. In addition, samples from public houses were more similar to samples from public buildings (distance = 0.36) than if these samples were compared to samples from ICU floors (distance = 0.65). Likewise, samples from medical devices were more similar to samples from ICU workplaces (distance = 0.37) in comparison to samples from floors (distance = 0.6). Moreover, different sampled surface materials showed defined microbial compositions as microbial communities on polymers, metals and tiles were distinct (distances > 0.6) from those found on wooden surfaces, but more similar to samples from stone surfaces (distances < 0.4). An approach (BIO-ENV with Spearman rank correlations compared to Euclidean distances) that correlated microbial community structure with environmental parameters like the geographic location, room architecture and microclimate conditions showed higher correlations of samples with latitude, longitude and the sea level (0.9425) than temperature, humidity and room variables like the surface area, room height, or room volume (0.7518). Furthermore, most environmental parameters were negatively correlated as determined via Mantel tests with distance classes (e.g. humidity, temperature, geographic location). Only the volume of sampled rooms showed rough positive correlations between Mantel correlation statistics and distance classes. To visualize these correlations between the microbial community structure and the environment, correlations were explained by vectors on an NMDS ordination of the sampled communities with calculated ellipses per sampling category (Fig. 5D). This ordination showed not only distinct clusters for samples from tiles in private houses, sanitary environments of public houses, public buildings, ICU floors and ICU workplaces overlapping with medical devices, but also could explain the difference between the ICU and all other sampled built environments by e.g. its biogeography or microclimate.

A PICRUSt analysis was used to compare predicted functional properties (using KEGG) from the 16S rRNA gene amplicon dataset at a higher resolved level to the shotgun metagenomics single read analysis. In contrast to microbial diversity, predicted functional diversity revealed a minor stringency of clustering for samples from ICU environments from all other samples as calculated by a jackknife UPGMA bootstrapped tree (Supplementary Fig. S5A) and a PCoA plot based on Bray-Curtis distances (Supplementary Fig. S5B). MRPP, adonis and ANOSIM tests (Supplementary Table S6B) gave indications that the pattern was more explainable by the

sampling location than the level of confinement. Adonis tests even showed that a high proportion (51.5%) of the distances could be explained by the materials present in different built environments. Overall all samples were much more similar on functional than on microbial level as determined via two-sided two sample Student's t-tests. Hence, even respective maximal distances showed only slight dissimilarities between moderate controlled and uncontrolled (0.1), ICU workplaces and the private house (0.14), tables and floors (0.12), as well as polymer tiles and furnished wooden surfaces (0.15). In contrast to microbial diversity, functional properties could be correlated to a slightly higher magnitude to the room architecture (0.8645), than geographic location (0.8123) and room microclimate (0.7024), as also visualized by respective environmental vectors on an NMDS ordination of the sampled communities with calculated ellipses per sampling category (Supplementary Fig. S5C). In addition, a heatmap showed more distinct patterns for individual samples, than whole sample groups of respective built environments (Supplementary Fig. S5D). A LEfSe analysis (Fig. 6) identified functions like environmental adaptation, functions associated to phagocytosis, and many metabolic pathways of amino acid biosynthesis to be typical for the controlled built environment. Apart from that, cancer pathways, apoptosis, many degradation processes of xenobiotics, geraniol, limonene, pinene, naphthalene, bisphenol, chlorocyclohexane, chlorobenzene, and drug metabolism as well as other transporters were distinctive for the hospital environment of the ICU. On the contrary, uncontrolled environments were characterized by general carbon metabolisms like starch, sucrose, amino sugar, nucleotide sugar, carbonhydrate metabolism and processes involved in glycolysis and gluconeogenesis.

These observations were mainly in line with results of the single read analysis of the shotgun metagenomics dataset.

Certain functions could be associated to each category of sampled built environments.

In general, functional traits were higher redundant over all sampled indoor spaces compared to microbial profiles. This is represented in an ordination analysis where samples from the shotgun metagenomics dataset were brought into relations to other metagenomics data sets of the phyllosphere and rhizosphere of green plants, urban indoor air, and the human microbiome project (Supplementary Fig. S6). A comparison of all SEED annotations on the highest functional level over all sampled built environments revealed that most subsystems showed their highest

respective relative abundance in samples obtained from the private house (1917 functions), followed by the gowning area (1011) and the ISO 8 cleanroom (913). On the contrary, samples from public houses (604), the intensive care unit (328), and public buildings (172) showed a lower proportion of their maxima of relative abundances per functional subsystem.

Assigned functions to each sample group showed a high level of redundant information of basic cellular functions like carbonhydrate, amino acid, protein and DNA metabolism and synthesis of cofactors, vitamins, prosthetic groups and pigments etc. (Fig. 7 A and B). Only samples from the private house included more functional categories at the set cutoff (>0.5%). However, a more detailed LEfSe analysis of SEED annotations (Fig. 7C) revealed functions associated to Gram positive bacteria (Gram positive cell wall components, heme and hemin uptake and utilization in Gram positives), fatty acid metabolism (fatty acid lipids, isoprenoids, teichoic and lipoteichoic acid biosynthesis), DNA repair systems (DNA repair UvrABC system, DNA repair bacterial Rec FOR pathway, transcription repair coupling factor), and heatshock (heat shock dnaK gene cluster) as significant discriminative features of uncontrolled built environments. On the contrary functions associated with Gram negative bacteria (Gram negative cell wall components), iron acquisition (ferrichrome iron receptor, TonB dependent siderophore receptor, siderophore pyoverdine), oxidative stress, membrane transport and secretion (Ton and Tol transport systems, RND efflux system inner membrane transporter CmeB, Type III, IV, VI ESAT secretion systems), virulence (virulence disease and defense) and resistances (resistance to antibiotics and toxic compounds, multidrug resistance efflux pumps, cobalt zinc cadmium resistance protein CzcA) were identified to be representative in controlled and confined built environments.

Co-occurrence networks revealed associations of receptors with resistance proteins and efflux systems (TonB receptors and Acriflavin resistance protein, RND efflux systems; Type I restriction-modification systems with Na+/H+ antiporter), basic functions in DNA metabolism (DNA polymerase subunits, Transcription-repair coupling factor, Excinuclease subunit) with fatty acid metabolism (Long-chain-fatty-acid CoA ligase) and with sulfate metabolism (Sulfate permease, acyl-CoA synthetase, Retron-type reverse transcriptase). Overall a denser co-occurrence network of SEED functions could be calculated again for the category of uncontrolled than controlled built environments (Fig. 8 A and B).

Even better quality for interpretations of effects from microbial maintenance on the functional properties of selected microorganisms, were possible at the level of reconstructed genomes from the metagenomics dataset.

Genome centric analysis and binning of metagenomic sequences gave insights into changed functional properties of close related species in different microbial controlled built environments.

Many assigned bins were representative for a certain environmental category: *Exiguobacterium* and *Macrococcus* for uncontrolled built environments; *Arthrobacter* and *Janibacter* for public buildings and houses; *Enhydrobacter*, *Kocuria* and *Pantoea* for public and private houses; *Lactococcus* and *Staphylococcus* for private houses; *Leuconostoc* for the transition from private houses to the moderate controlled environment of the ICU; and finally *Propionibacterium*, *Pseudomonas* and *Stenotrophomonas* for controlled built environments of the ICU and the cleanroom facility. Most bins could be assigned to the genus of *Acinetobacter*, which was present in all sampled built environments. Hence, this allowed a comparison of changed functional properties from different maintained built environments for *Acinetobacter* related binned genomes. Especially on the level of virulence, disease and defense obvious differences were visible for those *Acinetobacter* species, which could be derived from a controlled or uncontrolled built environment. According to our observations the number of assigned functions related either to virulence, disease or defense almost doubled in controlled built environments of the cleanroom facility and the hospital environment of the ICU. While uncontrolled built environments of public buildings and public as well as private houses showed functions like: bacteriocins ribosomally synthesized antibacterial peptides, copper homeostasis and tolerance, beta-lactamases, multidrug resistance efflux pumps and further resistances to arsenic, cobalt-zinc-cadmium, chromium compounds, fluoroquinolones, as well as antibiotics and toxic compounds; *Acinetobacter* species from microbial confined and controlled built environments showed all these functions and additionally: invasion and intracellular resistance to vancomycin and zinc, aminoglycoside adenyltransferases, bile hydrolysis, the colicin V and bacteriocin production cluster, *Listeria* surface proteins (internalin-like proteins) and the virulence operon of *Mycobacterium* involved in DNA transcription, protein synthesis, as well as possibly involved in quinolinate biosynthesis. The only unique function associated to virulence,

disease and defense of *Acinetobacter* related genomes found in naturally uncontrolled built environments was tolerance to colicin E2.

A comparison of all annotated SEED functions of high quality bins with RAST (Overbeek *et al.*, 2014; Aziz *et al.*, 2008) showed a high proportion of functions associated to amino acid and carbonhydrates metabolism for naturally uncontrolled built environments (Fig. 9). In contrast, bins obtained from microbial controlled built environments of the cleanroom facility indicated a shift towards other functions like virulence, disease and defense. Especially bins from the cleanroom environment showed much more evenly distributed functional capabilities for all functional groups and beside virulence, disease and defense also a high proportion of functions associated to stress response.

Discussion

The comparative analysis of deeply sequenced metagenomes from built environments with varying degree of microbial confinement revealed clear patterns of the microbiome structure in dependence of environmental conditions and maintenance. Hence, public buildings and houses represented naturally uncontrolled built environments with only very little microbial confinement and characterized by diverse building surface materials, varying levels of occupancy, minor effects from surface maintenance such as cleaning and high overall microbial diversity. All these environmental conditions had influence on the microbial composition to different magnitudes. Obviously, respective public and uncontrolled indoor microbiomes mirrored the surrounding outdoor environments and were dominated by gram-positive bacteria associated to soil (*Nocardioides*, *Arthrobacter, Exiguobacterium sibiricum*, and *Microlunatus phosphovorus*), air (*Knoellia aerolata* and *Janibacter hoylei*), and plants (*Kocuria palustris*). In contrast, kitchen and dining surfaces of private houses showed not only high abundance of lactic acid bacteria commonly found in dairy products (*Lactococcus garvieae*), fermented foods (*Weissella confusa*) and on fruits and vegetables (*Leuconostoc mesenteroides*), but also species associated to the normal animal flora of mammals and related meat products (*Streptococcus parauberis, Staphylococcus vitulinus, Macrococcus caseolyticus*). Whereas uncontrolled built environments were dominated by gram-positive microbes usually associated to the outdoor environment (soil, plants, air) and processed food (meat, fruits, vegetables), controlled built environments revealed a high abundance of sequences assigned to mainly human associated microbes (*Propionibacterium acnes, Acinetobacter johnsonii*), well-known opportunistic pathogens (*Staphylococcus aureus, Stenotrophomonas maltophilia, Acinetobacter baumannii*) and a low proportion of potential beneficials (*Pseudomonas putida*). These human associated species (*Pseudomonas, Propionibacterium* and *Porphyromonas*) could be also identified as a discriminant feature for controlled built environments towards uncontrolled built environments as were viral sequences of humans (herpes virus and papilloma virus). Human viral sequences were recently discovered in a viability linked metagenomics analysis of a spacecraft assembly cleanroom operated by NASA (Weinmaier *et al.*, 2015). Hence, the authors of this study could reconstruct a low abundant human associated virus (human cyclovirus 7078A) and a phage of *Propionibacterium* (phage P14.4) from their dataset. In addition, not only bacteria but also eukaryotes like arthropods (mites) and insects (lices, cockroaches) were discriminative for these confined environments. The

diversity of arthropods found indoors was just recently described (Bertone *et al.*, 2016) and showed not only a high diversity of arthropods in our homes, but also the ubiquitous distribution of book lices indoors. However the impact of arthropods as disease vectors, generators of allergens, and facilitators of the indoor microbiome on built environments remains obscure.

Distinct patterns of microbes in different maintained built environments could be also discriminated by different functional capabilities. Hence, uncontrolled built environments colonized by robust gram-positive bacteria from primarily an outdoor source had to encode especially for functions to adapt to fluctuating environmental conditions like the microclimate (heatshock, fatty acid metabolism), UV radiation (DNA repair systems), and nutrient availability (heme and hemin uptake and general processes of carbon metabolism and steps involved in glycolysis and gluconeogenesis). Detected functions associated to teichoic and lipoteichoic acid biosynthesis could be an obvious link to the predominance of gram-positive cells in this naturally uncontrolled built environment.

Opposed to this, the constant moderate microclimate of controlled built environments selected for host associated gram-negative bacteria highly influenced by humans. The close association to a eukaryotic host in this environment resulted in discriminant features for e.g. iron acquisition. Regular and strict cleaning procedures directed the microbiome to encode for functions associated to oxidative stress in combination with functions for membrane transport, secretion and apoptosis to gather nutrients from a highly competed oligotrophic environment, that was just recently described as a wasteland for microbes (Gibbons, 2016). The identification of oxidative stress as a discriminant functional feature inside the cleanroom could be also due to the changed lifestyle towards facultative and obligate anaerobes as recently suggested by Weinmaier and co-workers (Weinmaier *et al.*, 2015). Regular attack with cleaning reagents and toxins on these microbial populations were encountered by increased functional capabilities to degrade xenobiotics, geraniol, limonene, pinene, naphthalene, bisphenol, chlorocyclohexane, chlorobenzene, drug metabolism and an overall higher level of virulence, disease (cancer pathways), defense and resistances. Moreover, the detection of features from gram-positive (ESAT secretion systems), as well as gram-negative bacteria (Ton and Tol transport systems) and other functions like iron acquisition through siderophores and pyoverdine as well as the RND efflux systems could be an indication for a higher proportion of pathogens inside these controlled built environments. Hence, an increased diversity of functions associated to this category could be observed for *Acinetobacter* related species present in all sampled built environments. The

observation of a much broader functional repertoire for bins obtained from the cleanroom environment, might be due to constant exposition of microbes to the oligotrophic, nutrient poor and extreme conditions. Based on that, a flexible functional adaptation in this clean environment might be auxiliary for outlasting unfavorable conditions and general survival of its microbiome.

Hence, all this discriminative features on taxonomical and functional level resulted in clear separated microbial communities even on higher resolutions per sample as revealed via 16S rRNA gene amplicon sequencing. Despite known disadvantages of a targeted approach via amplicon sequencing (e.g. PCR biases) compared to the untargeted approach of shotgun metagenomics, the amplicon analysis very much supported observations of the shotgun metagenomics dataset. Likewise, this analysis clearly revealed decreasing levels of microbial diversity with an increase in microbial confinement of a built environment.

However, microbial confinement was not the sole driver of the microbial community structure as indicated by correlations with biogeography, a room's usage, architecture, or the microclimate. The ICU environment was characterized by a high room temperature and very low relative air humidity, which could explain the dissimilarity to other built environments as seen in several ordination analyses. Notable, low levels of air humidity in a room might lead to adverse health aspects. Lowen and co-workers showed that dry air could increase the infection potential of viruses (Lowen *et al.*, 2007) due to a reduced functionality of the human nasal mucosa to fend off infectious viruses (Arundel *et al.*, 1986; Mäkinen *et al.*, 2009; Williams *et al.*, 1996). Hence, the microclimate in the ICU should be reconsidered for a healthier immune system of its occupants in the presence of facilitated microbial growth conditions (temperature) and the changed functional capabilities of its microbiome.

Our study design might have been inappropriate to distinguish between distinct effects of all diverse drivers of the microbiome. However, similar influences on the microbiome inside offices were recently presented by (Chase *et al.*, 2016), who concluded that different microbial profiles on various building materials could be accounted more to a different human interaction with these materials than respective effects on the microbiome structure by physical or chemical properties. Hence, the unique rank of samples from the ICU environment in our study e.g. on the level of beta diversity, could be also derived from the manner how medical personal and patients differently interact with floors, workplaces and medical devices. In addition, the technical differences (sequencing platform and sampling equipment) of the ICU dataset could have been much more responsible for observed differences compared to other sample categories than actual

biological effects as was shown in a meta-analysis of the microbiology of the built environment (Adams *et al.*, 2015). Aware of these unfavorable attributes, we tried to reduce potential technical effects (sampling procedures, DNA extractions, sequencing run effects) as well as distortive environmental effects (sampling season and room locations) by a consistent experimental design and numerous controls throughout the study (Adams *et al.*, 2015; Chase *et al.*, 2016). PMA treatment of samples was suitable to mask potential contaminants of reagents and showed the high proportion of intact cells in public buildings as well as public and private houses. Therefore, in contrast to many other studies describing the microbiology of built environments, our data interpretations might be not only applicable for the total, but also the potential viable microbiome throughout these different sampled indoor environments.

Our comparative metagenomics analysis of different controlled and uncontrolled built environments revealed altered functional capabilities and profiles of its microbiome. These changes could be associated to some extent also to obvious environmental differences of sampled built environments. Microbial confinement of an indoor environment leads to different environmental conditions for microbes. Hence, isolation from the outdoor environment increases for instance the relative abundance of shed skin cells and associated human microbiota (Weinmaier *et al.*, 2015), but also decreases the proportion of beneficial plant associated microbes. Similarly, the higher proportion of Archaea in controlled built environments, could be also due to their close association to the human body (Probst *et al.*, 2013; Moissl-Eichinger, 2011). On the other hand, the high proportion of viruses in the ICU are obviously connected to the health status of its occupants, while their abundance in public buildings could be explained by the high proportion of sampled sanitary environments and a high frequentation by many people in general.

Hence, a higher proportion of human microbiota could result in a higher potential of the surrounding microbial world to influence our immune system and affect our health inside buildings either positively or negatively. The Microbiome structure was shown to be already triggered at the level of architecture, microclimate and maintenance inside confined areas (Kelley and Gilbert, 2013; Kembel *et al.*, 2012; Lax *et al.*, 2014; Moissl-Eichinger *et al.*, 2015). Furthermore the low exchange with the outdoor environment reduces microbial diversity inside buildings (Meadow *et al.*, 2014) and could weaken its overall stability (Kennedy *et al.*, 2002). However, we have to face increasing problems of confined built environments and their

associated health risks especially for immune compromised patients (Lax and Gilbert, 2015). Hence, confinement of a built environment should be limited to defined areas. In all other areas of a built environment, air conditioning systems that provide a constant but inconvenient microclimate and limit the exchange with outdoor air should be reconsidered. Building materials should be diverse to allow a higher microbial diversity (Chase *et al.*, 2016). In addition, materials of natural sources are preferred to allow the transfer of already intact and stable microbial communities to a new built environment. An easy way to increase microbial diversity inside buildings is the introduction of green ornamental plants and microbiomes from outdoors (Mahnert *et al.*, 2015).

The presence of highly diverse and stable beneficial designed microbiomes inside healthy buildings could result in lower proportions of nosocomial infections in hospitals, or a reduced risk to develop atopic diseases or even finally lower costs for microbial maintenance indoors.

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Author Contributions

Study design: AM, CM-E and GB. Sampling and sample processing: AM. Data analysis AM, MZ, TR. Wrote the manuscript: AM. All authors read and improved the final manuscript.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figures and Tables for:

The altered microbiome of confined built environments

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Running title: Comparative metagenomics of controlled and uncontrolled built environments

Keywords: controlled and uncontrolled indoor microbiomes, cleanrooms, intensive care units, public buildings, private houses, wildlife park, shotgun metagenomics, 16S rRNA gene amplicons, viability

Table 1: Summary on some environmental parameters of samples and the dataset.

Environmental parameters	public buildings (L)	public houses (P)	private houses (F)	intensive care unit (ICU)	gowning area (UR)	cleanroom (CR)
shotgun metagenome samples	2	2	2	1	1	1
16S rRNA gene amplicon samples	18	6	8	24	2	3
restriction level	non		partly	moderate	high	
occupancy type	uncontrolled		restricted	restricted	controlled	
surface material	polymer, concrete, tiles, wood	tiles, wood	polymer, tiles, stone	polymer, furnished wood, metals	polymer (antistatic, dissipative epoxy resin)	
location	floors			floors, workplaces, medical devices	floors	
building setting	rural			suburban	urban	
maintenance	conventional			humidity, temperature	(particles), humidity, temperature, (electromagnetics, electrostatics)	particles, humidity, temperature, electromagnetics, electrostatics
cleaning	mechanically (broom)	natural soaps	all-purpose cleaners	all-purpose cleaners, surface disinfectant, sanitary cleaners	isopropanol, JAMINAL PLUS, KLERCIDE-CR	isopropanol, JAMINAL PLUS, KLERCIDE-CR, vapor phase H_2O_2
purpose	education	accomodation	residence	medical care	changing garment	spacecraft assembly
characteristics	reduced interaction of occupants with the outdoor environment and its influences	sanitary area and kitchen included	kitchen included, resident and dog (pet)	medical care of patients, microbes and viruses	gowning area	HEPA air filtration, special garment, microbial control
sampled surface [m^2]	43	46	25	<1	38	169
room size [m^2]	43	46	25	75	38	169
room height [m]	3	3	3	3	3	8
room volume [m^3]	142	139	82	225	113	1349
room air humidity [%]	62	65	60	32	55	55
room air temperature [°C]	19	18	19	24	22	22
geographic location latitude	53.950258	53.948030	53.953343	47.081353	45.079675	45.079675
geographic location longitude	10.031095	10.026079	10.034461	15.465090	7.608334	7.608334
sea level [m]	28	28	31	394	279	279

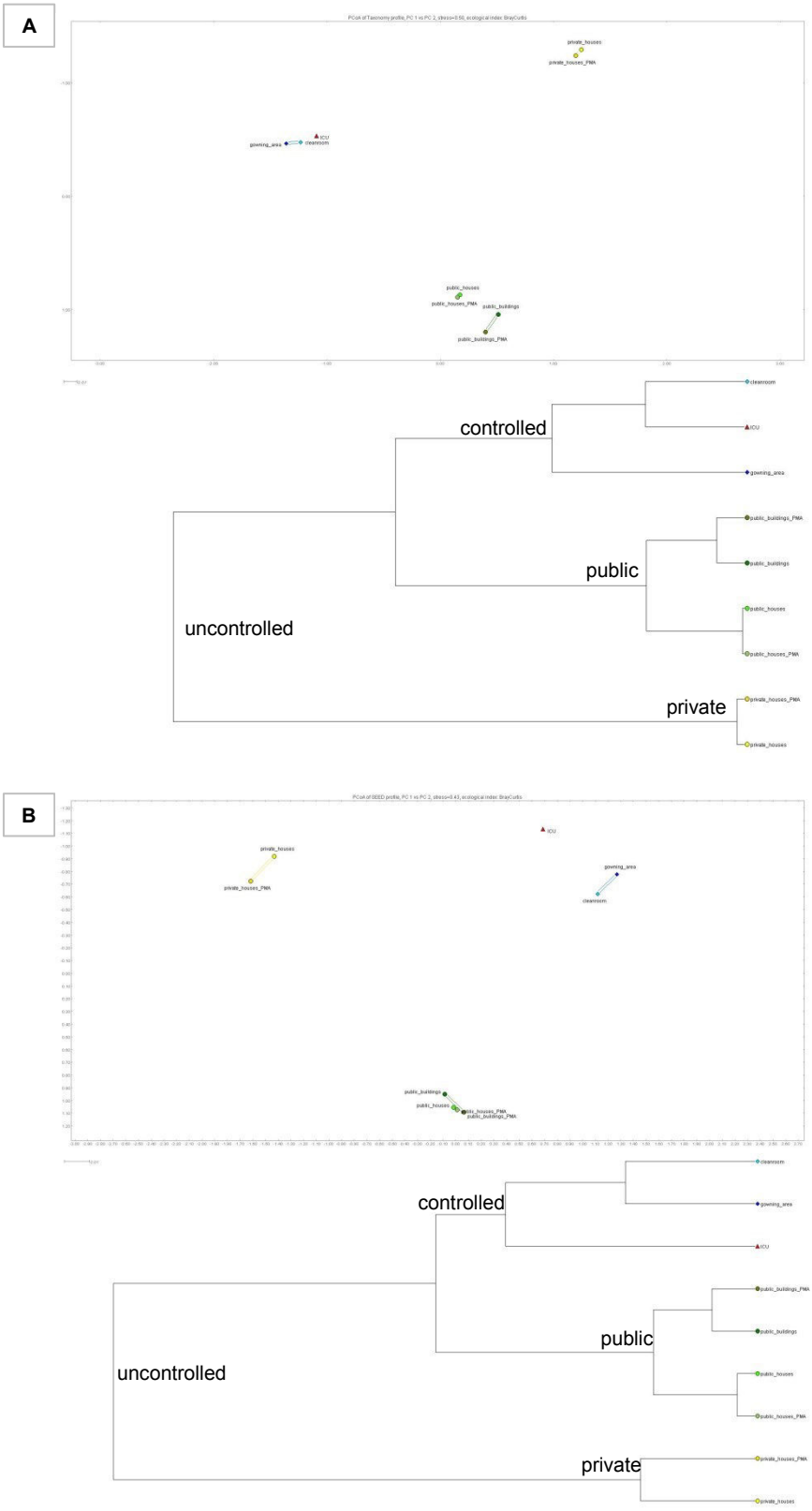


Fig. 1: A) Top: PCoA of taxa (blastx vs. NCBI nr reads, excluding unassigned, normalized, linear); Bottom: UPGMA tree of taxa (blastx vs. NCBI nr reads, excluding unassigned, normalized, linear). **B)** Top: PCoA of SEED annotations (blastx vs. NCBI nr reads, excluding unassigned, normalized, linear); Bottom: UPGMA tree of SEED annotations level 1 (blastx vs. NCBI nr reads, excluding unassigned, normalized, linear).

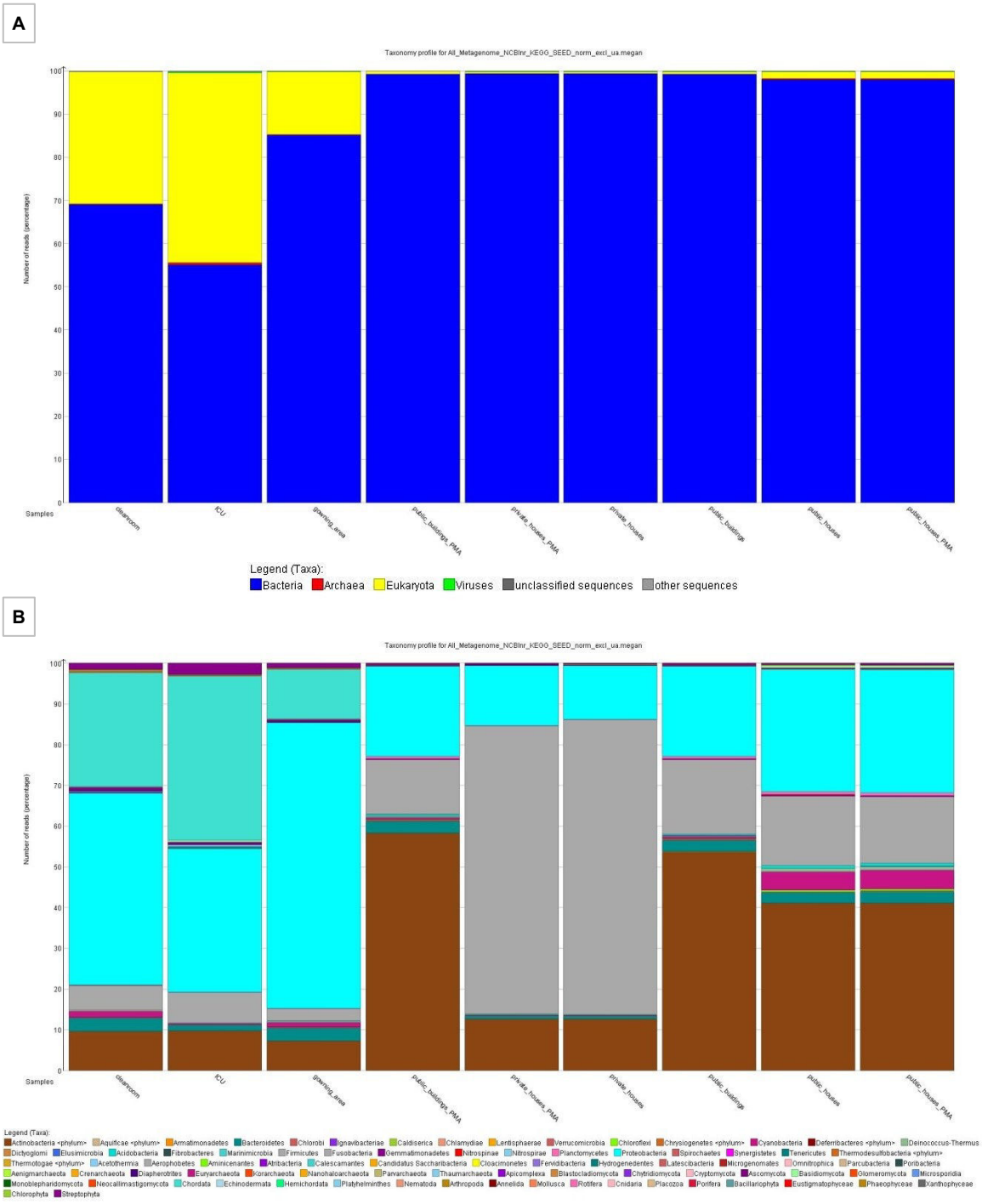
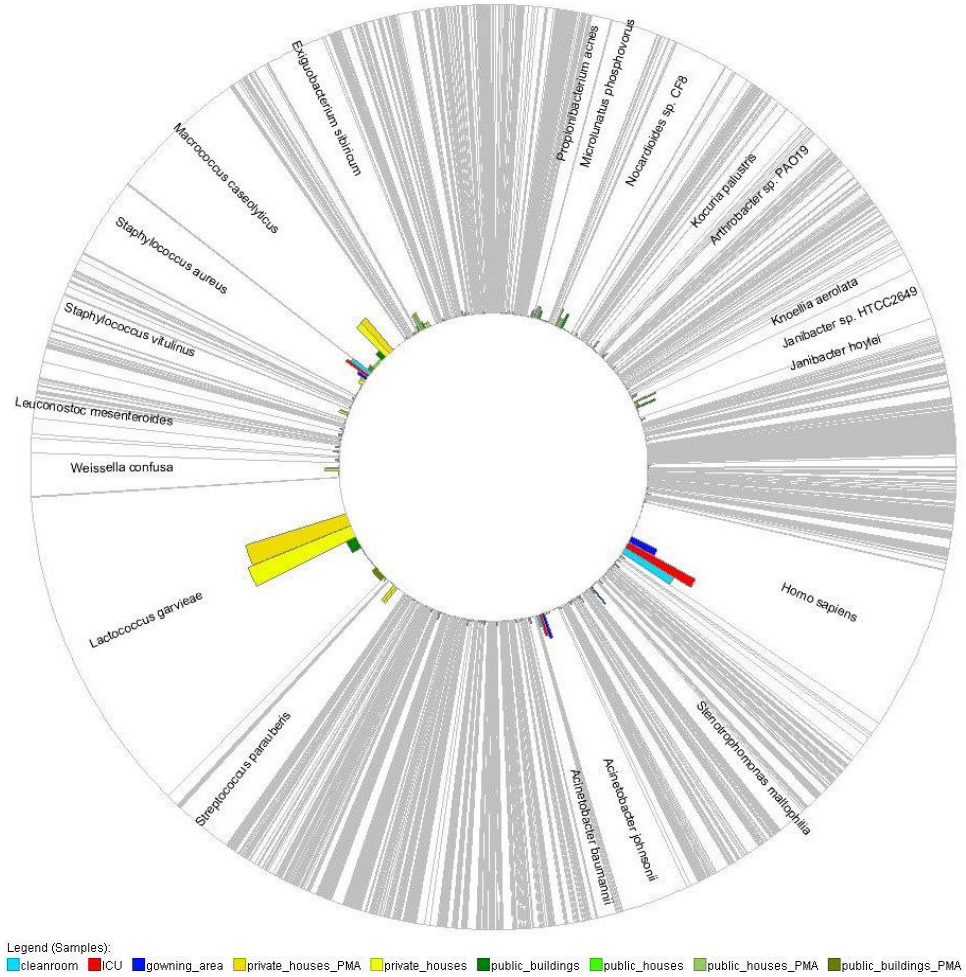


Fig. 2: A) Single reads blastx (rapsearch and diamond) vs. NCBI nr. superkingdom level (derived from MEGAN, excluding unassigned reads, normalized data set); **B)** Single reads blastx (rapsearch and diamond) vs. NCBI nr. phylum level (derived from MEGAN, excluding unassigned reads, normalized data set)



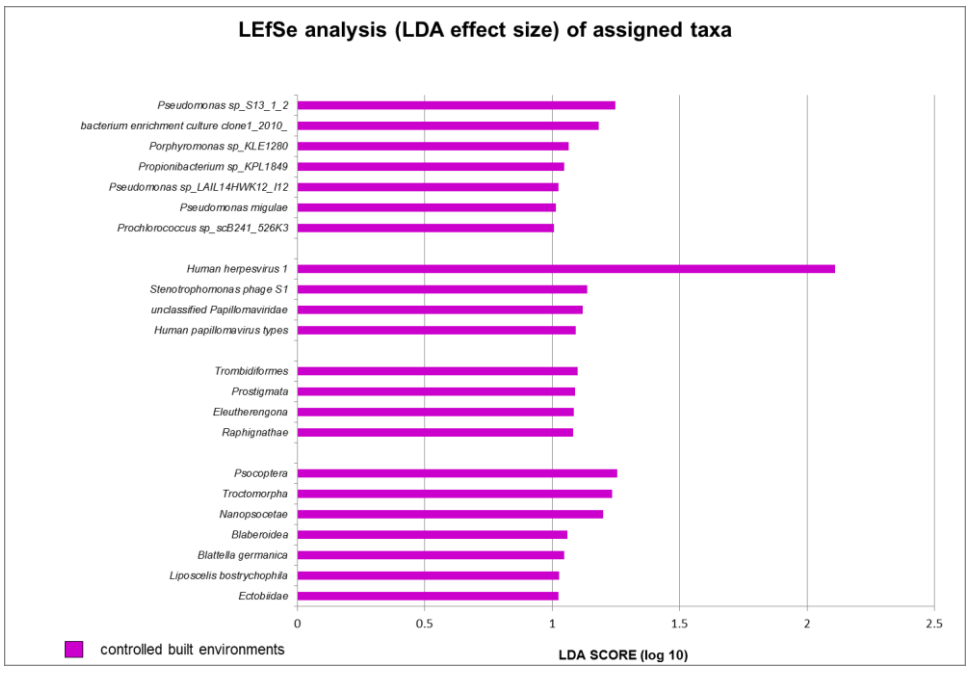


Fig. 2: C) Space filling radial chart of taxa (species level, excluding unassigned reads, normalized, percentage) assigned (blastx NCBInr, diamond and rapsearch) to different built environments (MEGAN); **D)** LEfSe analysis (LDA effect size) on taxa (according to NCBInr database) of single reads from metagenomes of controlled (ICU, gowning area, cleanroom) and uncontrolled (public buildings, public and private houses) built environments with the following parameters: per-sample normalization to 1M, factorial Kruskal-Wallis test among classes (alpha = 0.01), pairwise Wilcoxon test between subclasses (alpha = 0.01), threshold for the LDA score (1.0), strategy for mulit-class analysis (all-against-all, more strict).

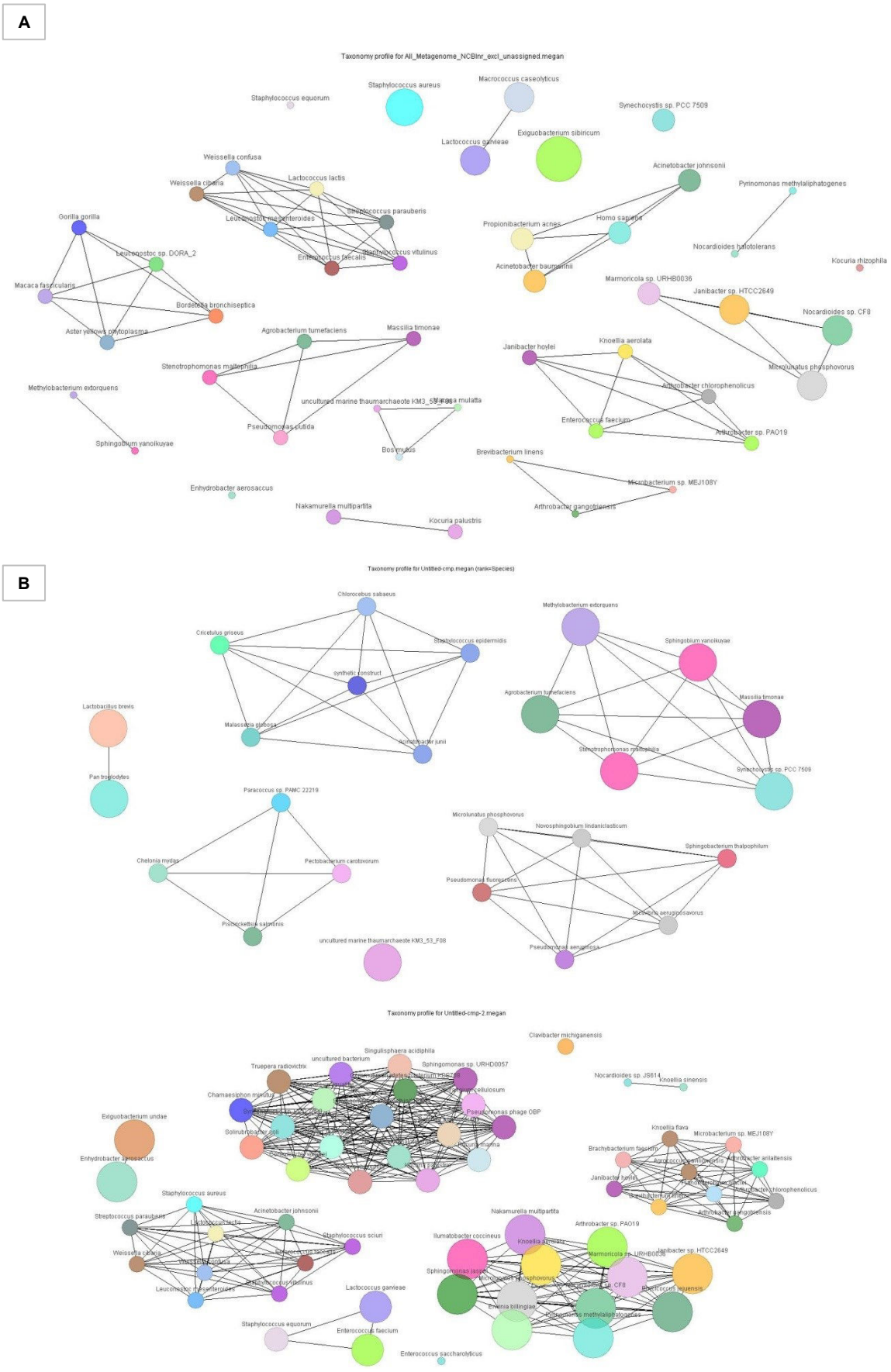


Fig. 3: A) Co-occurrence network of all taxa (blastx vs. NCBI nr reads, excluding unassigned, normalized, linear, threshold 1%, min. prevalence 10%, max. prevalence 90%, probability 70%); **B)** Comparative co-occurrence network of taxa (species level, blastx vs. NCBI nr reads, excluding unassigned, normalized, linear, threshold 0.5%, min. prevalence 10%, max. prevalence 90%, probability 70%) from uncontrolled (top) and controlled (bottom) built environments.

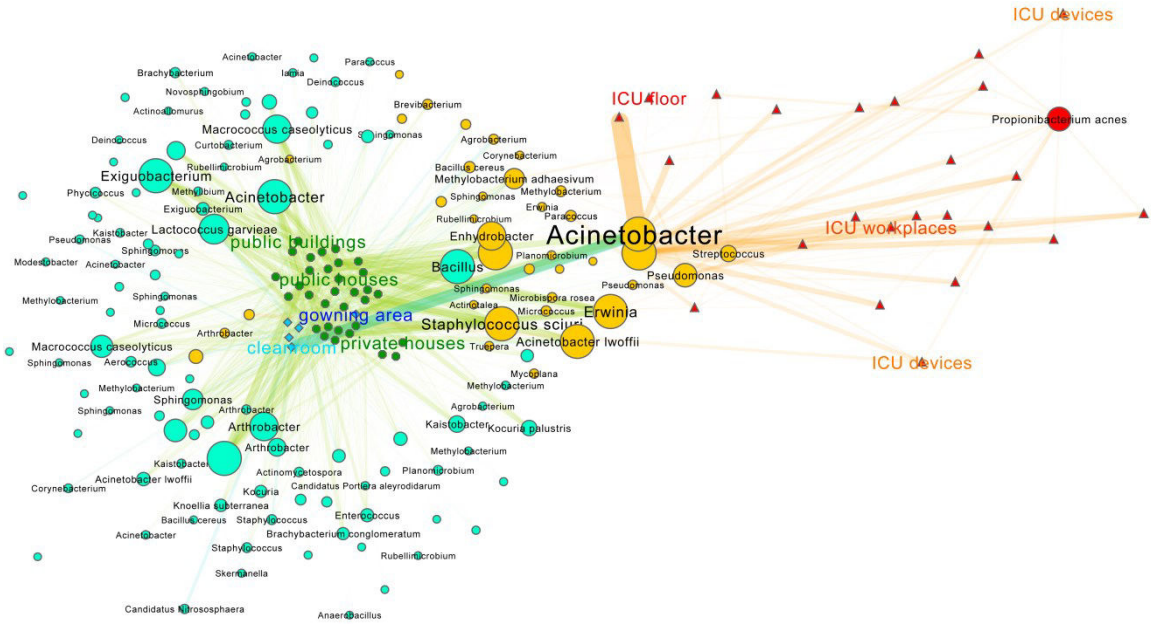
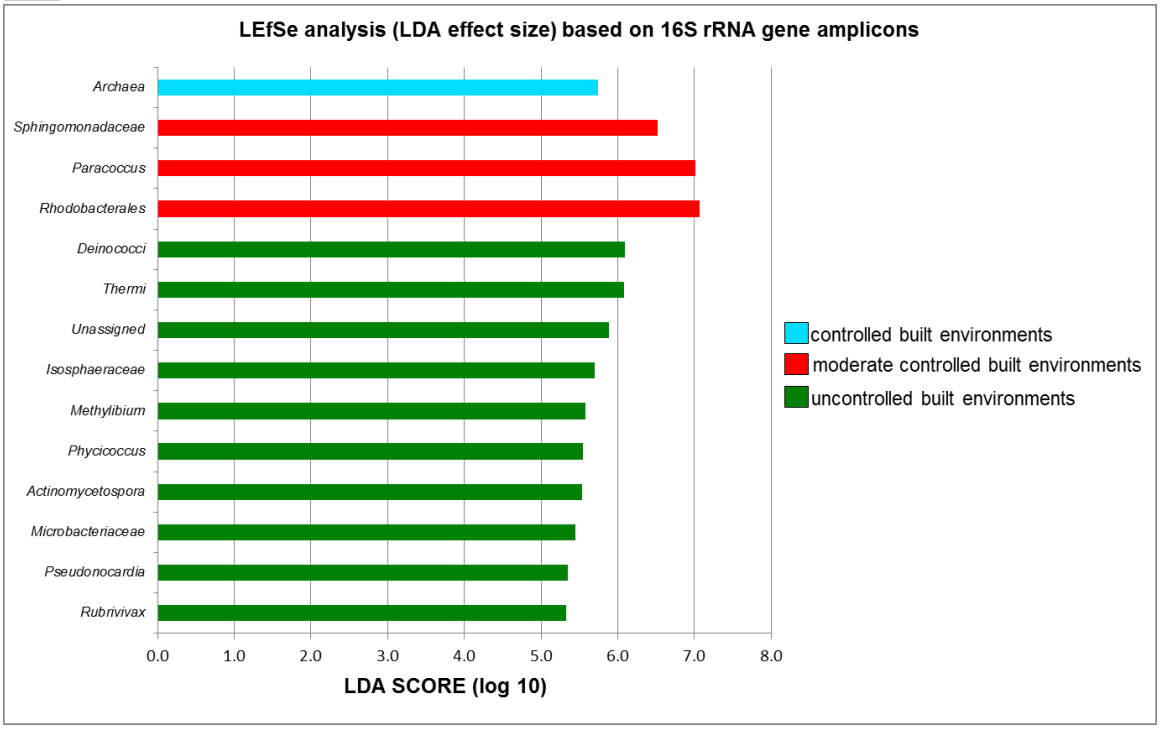


Fig. 4: Core OTU network, nodes represent core OTUs, node size indicates relative abundance, node labels show taxonomic assignments at genus level, edges are weighted, colors represent controlled, moderate controlled and uncontrolled built environments.

A



B

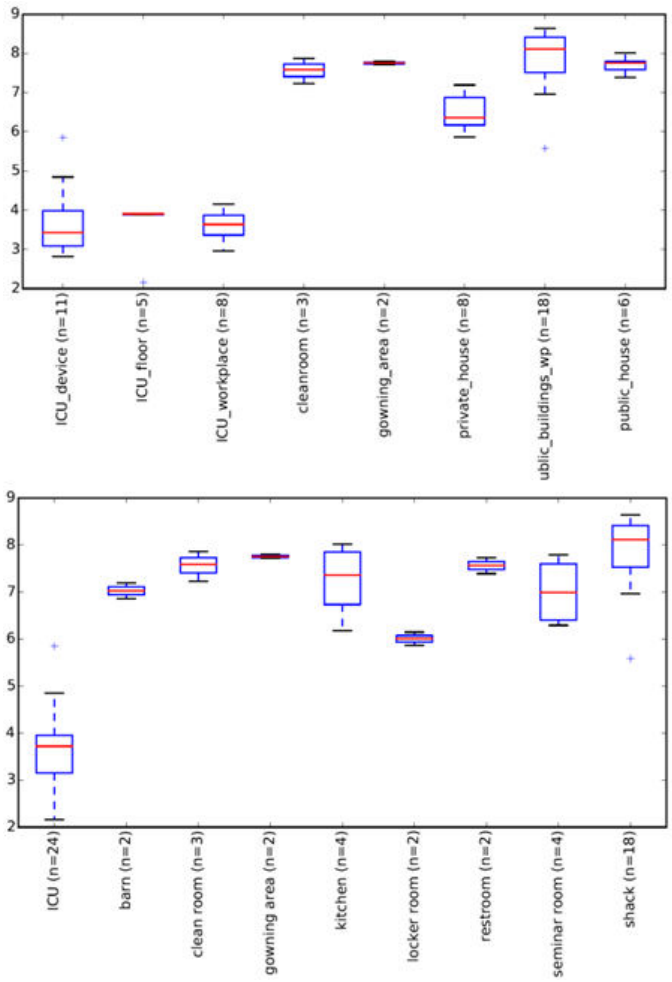


Fig. 5: A) LEfSe analysis (LDA effect size) on 16S rRNA gene amplicons of controlled (gowning area, cleanroom), moderate controlled (ICU) and uncontrolled (public buildings, public and private houses) built environments with the following parameters: per-sample normalization to 1M, factorial Kruskal-Wallis test among classes (alpha = 0.05), pairwise Wilcoxon test between subclasses (alpha = 0.05), threshold for the LDA score (2.0), strategy for mulit-class analysis (all-against-all, more strict); **B)** Alpha diversity of 16S rRNA gene amplicons based on Shannon index per built environment type (left, grouping) and indoor space (right).

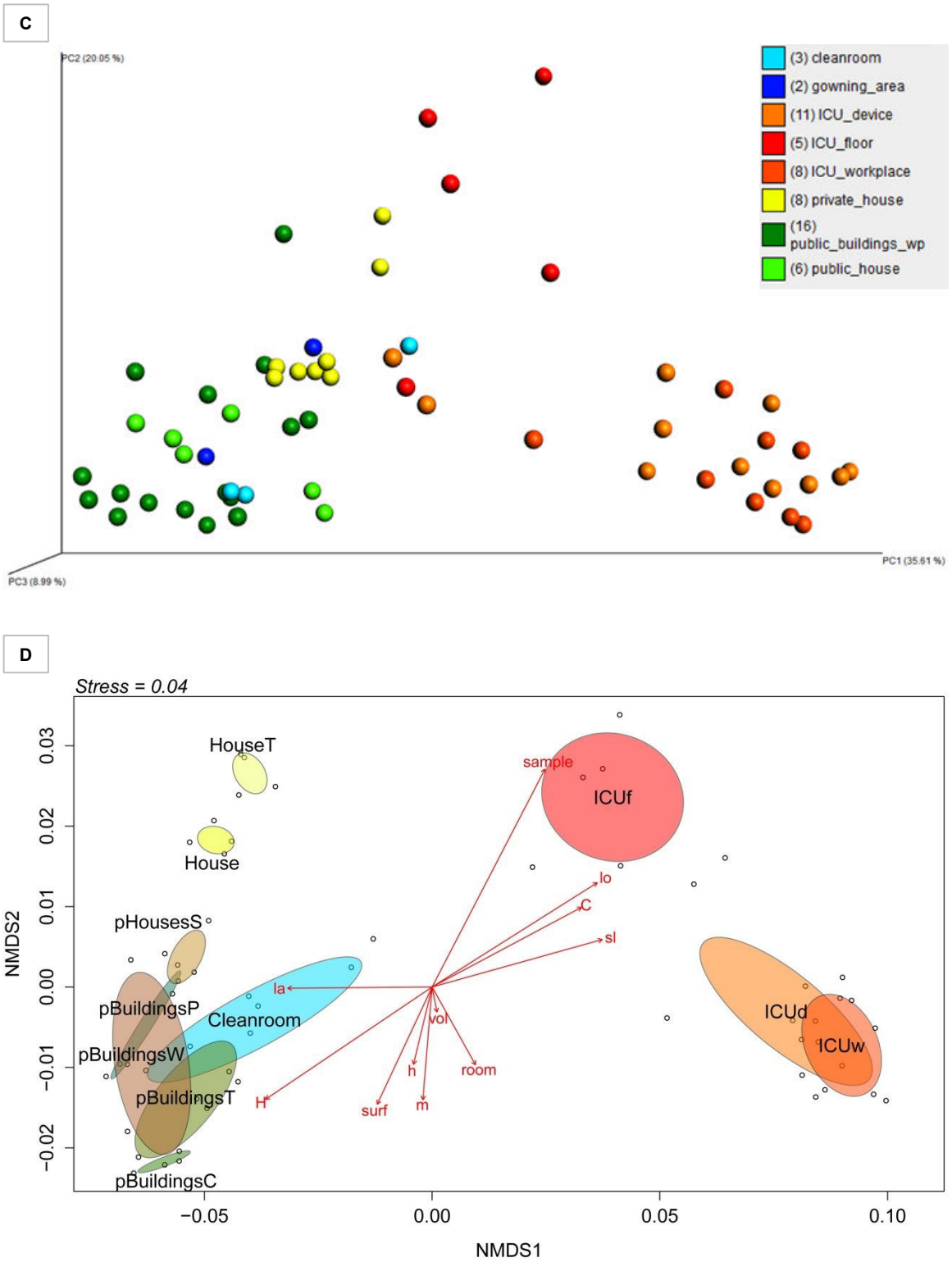


Fig. 5: C) Beta diversity presented as a PCoA of 16S rRNA gene amplicons based on weighted unifrac distances at a depth of 2052 sequences per sample; **D)** Beta diversity presented as NMDS of 16S rRNA gene amplicons based on Bray-Curtis distances with superimposed vectors representing Spearman correlations of measured environmental variables (BIO-ENV) based on Eucledian distances. Ellipses for sample groupes were calculated for the following categories: public buildings with concrete (pBuildingsC), tiles (pBuildingsT), polymers (pBuildingsP), wooden floors (pBuildingsW); public houses (pHouses) with samples from sanitary areas (pHousesS); private houses (House) with tiled floors (HouseT); ICU devices (ICUd), floors (ICUf) and workplaces (ICUw), samples of the cleanroom facility (Cleanroom).

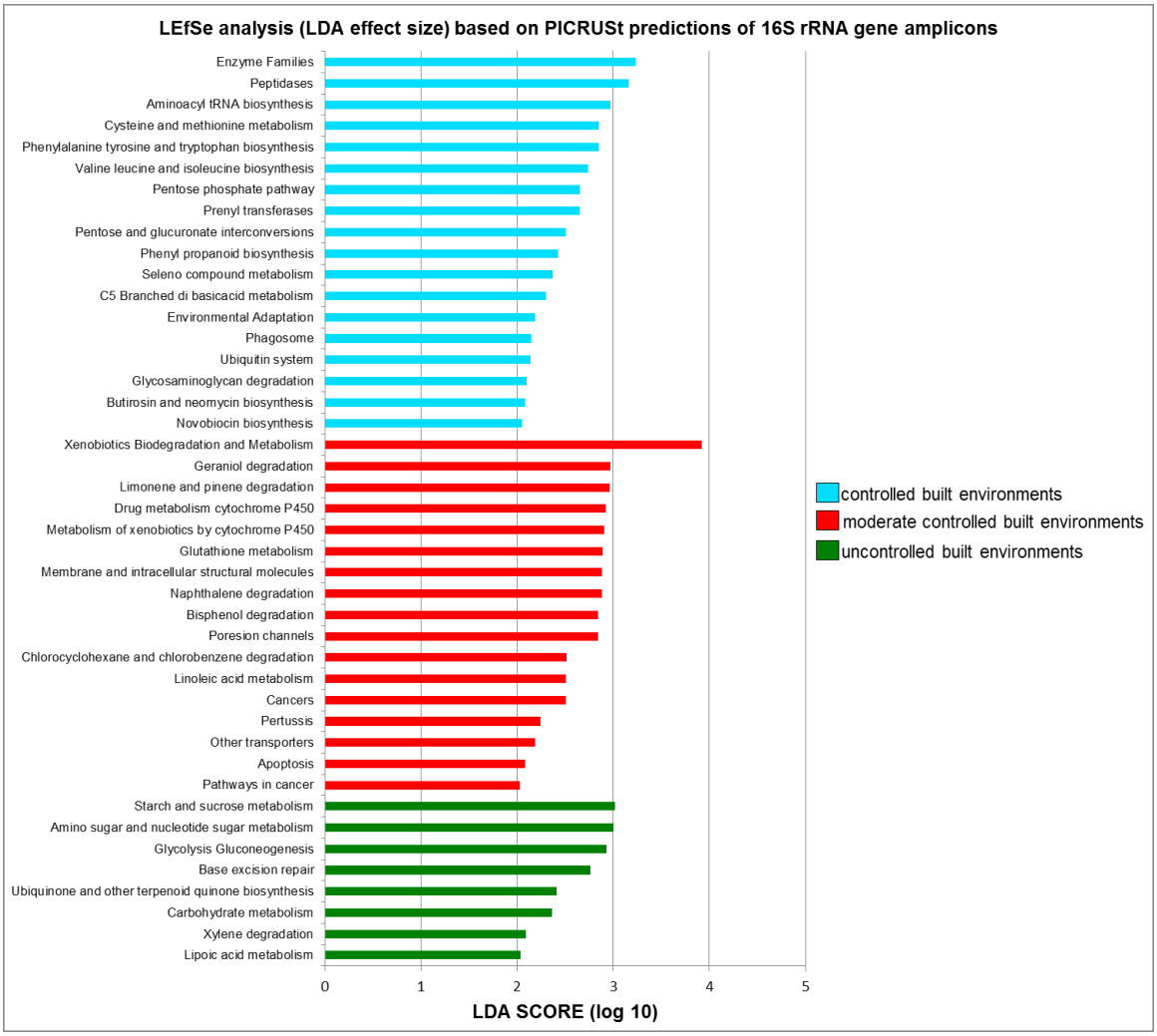


Fig. 6: LEfSe analysis (LDA effect size) on PICRUSt predicted functions of 16S rRNA gene amplicons of controlled (gowning area, cleanroom), moderate controlled (ICU) and uncontrolled (public buildings, public and private houses) built environments with the following parameters: per-sample normalization to 1M, factorial Kruskal-Wallis test among classes (alpha = 0.05), pairwise Wilcoxon test between subclasses (alpha = 0.05), threshold for the LDA score (2.0), strategy for mulit-class analysis (one-against-all, less strict)

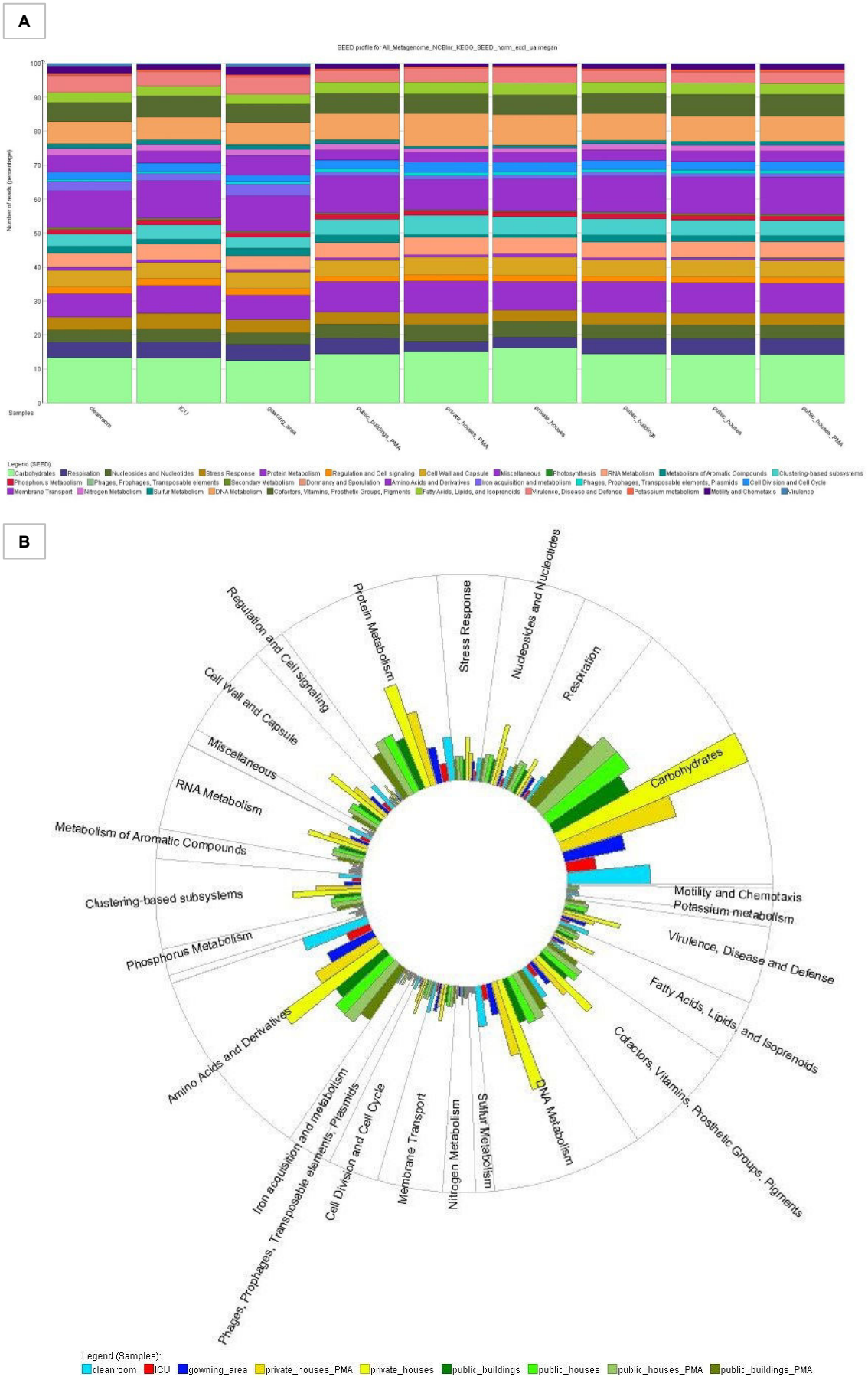


Fig. 7: A) Single reads blastx (rapsearch and diamond) vs. NCBI nr. SEED level 1 (derived from MEGAN, excluding unassigned reads, normalized data set); **B)** Space filling radial chart of SEED annotations on level 1 (species level, excluding unassigned reads, normalized, percentage) assigned (blastx NCBInr, diamond and rapsearch) to different built environments (MEGAN).

C

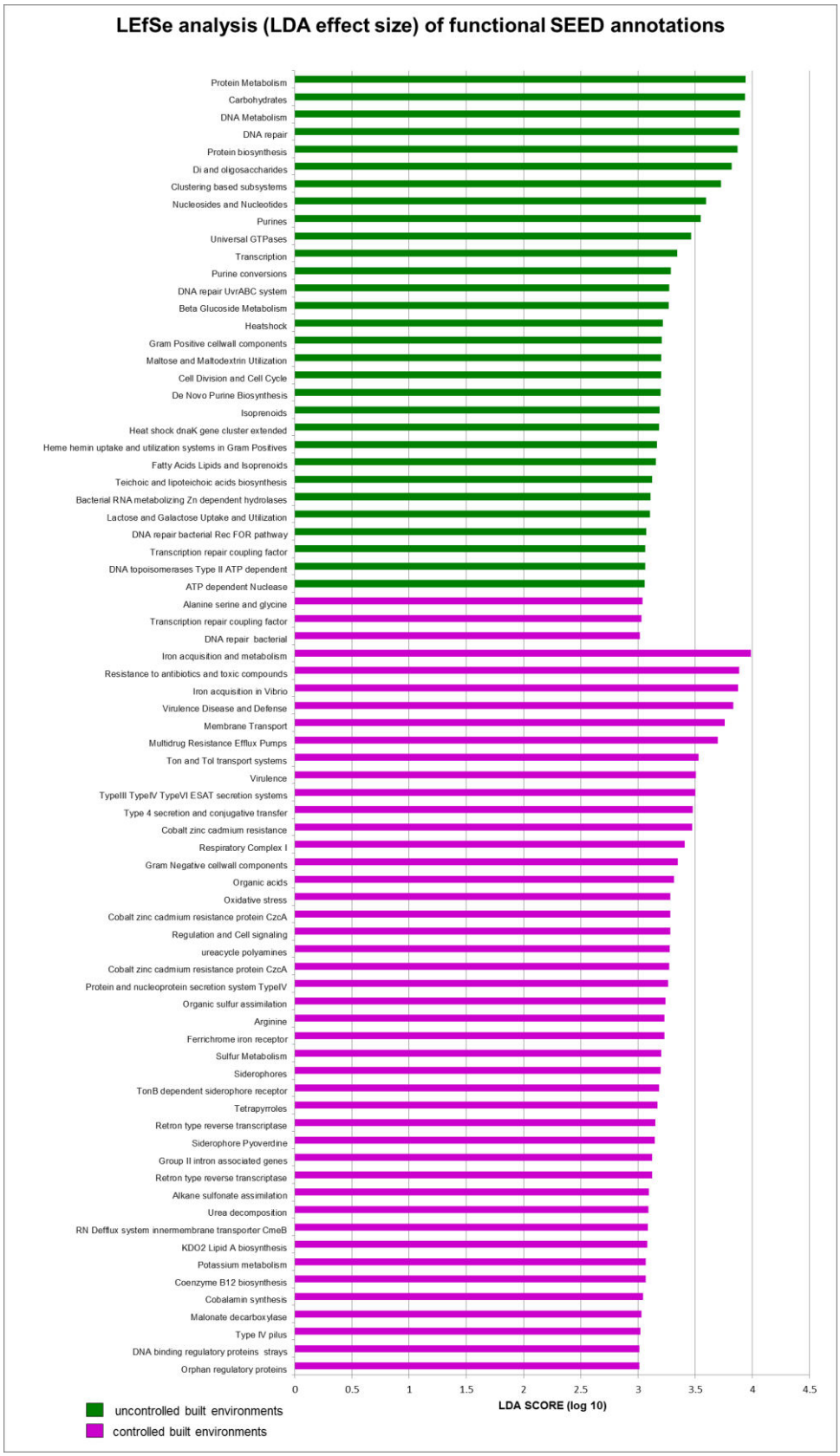


Fig. 7: C) LEfSe analysis (LDA effect size) on functions (according to SEED database) of single reads from metagenomes of controlled (ICU, gowning area, cleanroom) and uncontrolled (public buildings, public and private houses) built environments with the following parameters: per-sample normalization to 1M, factorial Kruskal-Wallis test among classes (alpha = 0.05), pairwise Wilcoxon test between subclasses (alpha = 0.05), threshold for the LDA score (3.0), strategy for mulit-class analysis (all-against-all, more strict).

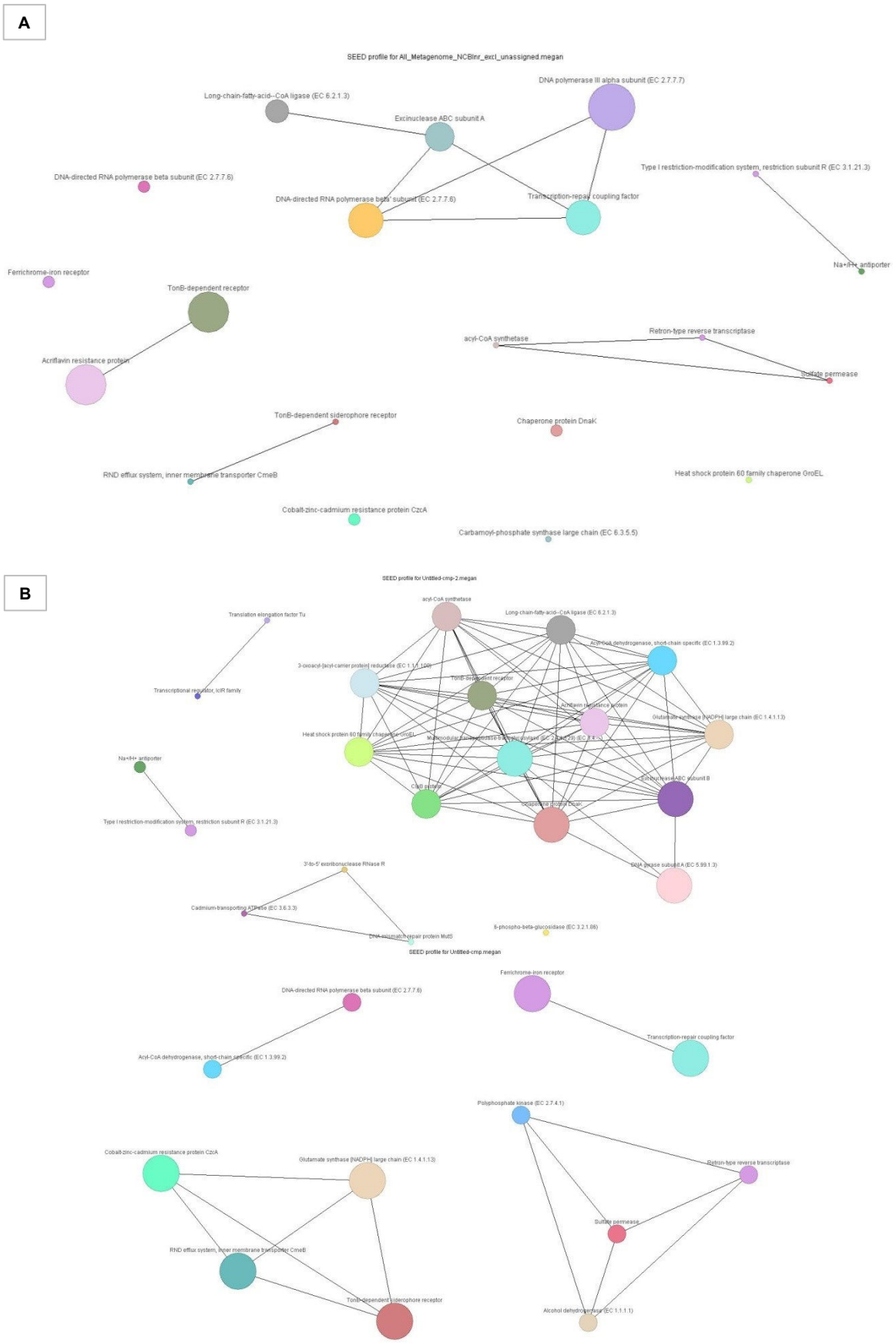


Fig. 8: A) Co-occurrence network of SEEDs (level 3, blastx vs. NCBI nr reads, excluding unassigned, normalized, percentage, threshold 0.4%, min. prevalence 10%, max. prevalence 90%, probability 70%); **B)** Comparative co-occurrence network of SEEDs level 3 (blastx vs. NCBI nr reads, excluding unassigned, normalized, linear, threshold 0.3%, min. prevalence 10%, max. prevalence 90%, probability 70%) of uncontrolled (top) and controlled (bottom) built environments.

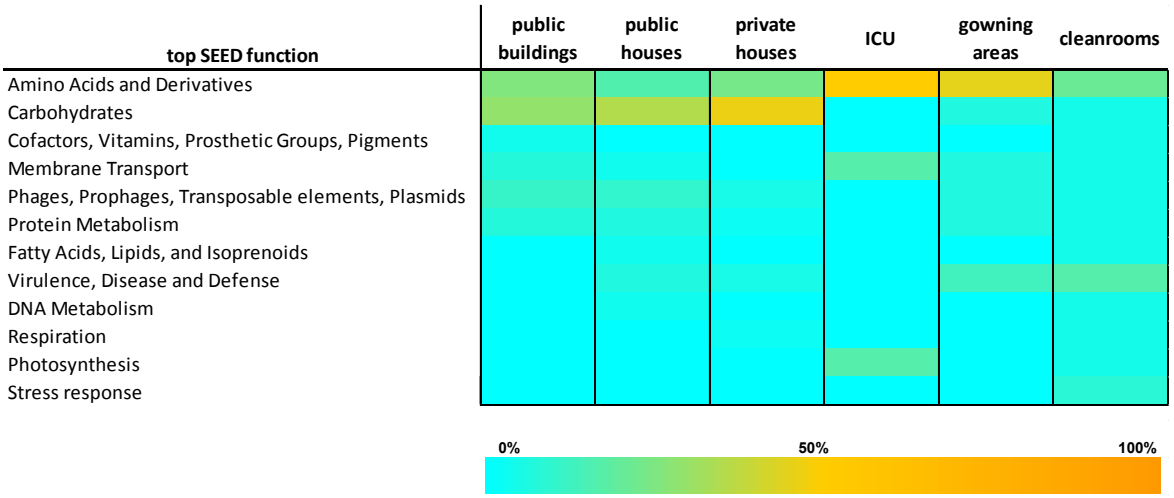


Fig. 9: Relative proportions of annotated SEED functions with RAST for high quality bins from the metagenomics dataset of all sampled built environments.

Supplementary Figures and Tables for:

The altered microbiome of confined built environments

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Running title: Comparative metagenomics of controlled and uncontrolled built environments

Keywords: controlled and uncontrolled indoor microbiomes, cleanrooms, intensive care units, public buildings, private houses, wildlife park, shotgun metagenomics, 16S rRNA gene amplicons, viability

Table S1A: A list of cleaning and disinfection reagents applied for various surfaces and purposes in the sampled built environments.

built environment											
public buildings (L)		public houses (P)		private houses (F)		intensive care unit (ICU)		gowning area (UR)		cleanroom (CR)	
floors	mechanically (broom)	floors	natural soaps	floors	all-purpose cleaners	hand desinfection	Desderman pure Desmanol pure Descoderm Skinman Soft Protect Sterilium classic pure Sterillium LSG	surfaces	isopropanol 70% JAMINAL PLUS KLERCIDE-CR	surfaces	isopropanol 70% JAMINAL PLUS KLERCIDE-CR
						skin antiseptic	Kodan forte (colored) Kodan forte (colorless) Betaseptic Betaisodona standardized solution	devices and products	isopropanol 70%	devices and products	isopropanol 70% vapor phase H_2O_2 autoclaving
						mucosal antiseptic	Octenisept solution Betaisodona standardized solution				
						surface desinfection	Incidin Plus				
						rapid desinfection (containing alcohol)	Incidin Liquid				
						rapid desinfection (nonalcoholic)	Acryl-des desinfection tissues Acrylan				
						desinfectant for instruments (manually preparation)	Gigasept Instru AF Sekusept plus Sekusept active				

Table S1B: A list of cleaning and disinfection reagents including the exposure time applied for certain cases in the ICU at the state hospital in Graz, Austria.

disease / germ	range of application		
	hand desinfection	surface desinfection	
		rapid desinfection	routine desinfection
Norovirus	Desderman pure (30 sec) Desmanol pure (30 sec)	Incidin liquid (10 min)	Incidin active 1% (1 hr)
Adenovirus	Desderman pure (30 sec)	Incidin liquid (10 min)	Incidin plus 1% (1 hr)
Rotavirus	Desderman pure (30 sec) Desmanol pure (30 sec) Descoderm (30 sec)	Incidin liquid (5 min)	Incidin plus 0.5% (1 hr)
Clostridium difficile	standard desinfectants according to cleaning and desinfection protocols	Incidin active 2% (15 min)	Incidin active 1% (30 min)
gas gangrene	standard desinfectants according to cleaning and desinfection protocols (in-depth cleaning)		
tuberculosis	standard desinfectants applied 2 times according to cleaning and desinfection protocols	Incidin liquid (5 min)	Incidin plus 0.5% (1 hr)
MRE (MRSA, ESBL etc.) hepatitides, HIV influenza A, B measles, mumps Meningococci pertussis Salmonellae SARS varicella	standard desinfectants according to cleaning and desinfection protocols		

Table S2: Summary of all contigs and scaffolds after assembly of the shotgun metagenomics dataset.

Contigs >= 100 nt	Number	Total length	Average	N50	Median	Largest
CR8	8603965	2010442497	233	251	173	88313
UR	6781605	1285900173	189	169	148	77951
ICU	5846714	1194191177	204	204	162	152957
L	3719862	616578790	165	144	140	112186
L_plus	3595693	606816993	168	146	141	192834
P	3609403	583352985	161	144	140	260735
P_plus	3529598	569566003	161	145	140	260738
F	3013516	528935438	175	143	138	128864
F_plus	3103321	529882363	170	142	138	98570

Contigs >= 500 nt	Number	Total length	Average	N50	Median	Largest
ICU	98994	73962331	747	625	574	152957
CR8	468389	338833151	723	667	613	88313
UR	103191	129616359	1256	1523	713	77951
L_plus	50525	64061830	1267	1449	771	192834
P	31304	47448008	1515	2299	766	260735
P_plus	29900	44378695	1484	2206	748	260738
F	59104	91192370	1542	2084	870	128864
F_plus	54071	82752023	1530	2032	867	98570
L	50240	60661672	1207	1324	750	112186

Scaffolds >= 100 nt	Number	Total length	Average	N50	Median	Largest
ICU	5846470	1194230266	204	204	162	152957
CR8	8602774	2010603824	233	251	173	88313
UR	6779560	1286163090	189	169	148	101382
L_plus	3594500	606967359	168	146	141	258085
P	3608497	583483719	161	144	140	260735
P_plus	3528733	569693266	161	145	140	260738
F	3011212	529221167	175	143	138	128864
F_plus	3101251	530124777	170	142	138	98570
L	3718903	616698401	165	144	140	155114

Scaffolds >= 500 nt	Number	Total length	Average	N50	Median	Largest
ICU	98764	74008162	749	626	574	152957
CR8	467352	339066479	725	668	612	88313
UR	101444	130012967	1281	1592	711	101382
L_plus	49546	64308268	1297	1520	772	258085
P	30472	47613011	1562	2510	763	260735
P_plus	29106	44538821	1530	2402	744	260738
F	57173	91638271	1602	2264	872	128864
F_plus	52403	83164916	1587	2194	871	98570
L	49474	60867124	1230	1371	751	155114

Table S3: Summary on all quality reads of the shotgun metagenomics dataset.

samples	sequences (per each read)	sequence length	%GC	read	Phred Score (average quality per read; seq/q)
CR8	6.71E+07		44	1	>2.5E+07/37
				2	>2.0E+07/37
UR	5.35E+07		48	1	>1.8E+07/37
				2	>1.4E+07/37
ICU	3.30E+07		42	1	>1.6E+07/37
				2	>1.4E+07/37
L	3.03E+07		60	1	>8.0E+06/36
				2	>8.0E+06/36
L_plus	3.17E+07	50-150	62	1	>9.0E+06/36
				2	>8.0E+06/36
P	3.32E+07		60	1	>1.0E+07/36
				2	>7.0E+06/36
P_plus	3.28E+07		61	1	>9.0E+06/36
			60	2	>7.0E+06/36
F	3.97E+07		45	1	>1.4E+07/37
				2	>1.2E+07/37
F_plus	3.69E+07		46	1	>1.2E+07/37
				2	>1.2E+07/37

Table S4: Read statistics of the 16S rRNA gene amplicon dataset.

16S rRNA gene amplicons	
Number of samples	61
Number of OTUs (operational taxonomic units)	10814
Number of sequences	837216

Number of sequences per sample	
L5	225
L1.PMA	254
ICU.f.1	2052
ICU.f.5	2888
CR.7a	2928
ICU.f.4	4424
L8.PMA	4576
F2.PMA	5227
ICU.d.6	5308
F2	6444
ICU.d.11	6635
ICU.w.23	6778
ICU.w.18	7057
ICU.f.2	7168
UR.7	7255
L3.PMA	7307
L4.PMA	7543
ICU.w.17	7721
ICU.d.15	7841
P3	7920
L5.PMA	7965
ICU.d.9	8088
ICU.w.22	8221
ICU.d.13	8446
F3	8616
ICU.d.7	9243
ICU.w.24	9627
ICU.d.8	9628

Number of sequences per sample	
ICU.d.14	10228
ICU.d.10	10305
ICU.w.19	10504
F3.PMA	10846
ICU.f.3	10877
L1	10943
ICU.d.16	11185
ICU.d.12	11408
L6.PMA	12149
ICU.w.21	13147
P3.PMA	13454
ICU.w.20	14835
L3	14896
L9	15899
L7.PMA	16261
P1.PMA	18562
CR.7b	18888
P2.PMA	19242
L7	19359
L6	21036
P1	21321
L4	24486
F1	25455
F1.PMA	25670
L2	26425
UR	26913
P2	27974
L8	28344
F0	28566
L9.PMA	30456
F0.PMA	35436
CR.8	36930
L2.PMA	37831

summary of reads	
Minimum	225
Maximum	37831
Median	10504
Mean	13724.852
Std. dev.	9289.725

Table S5: Alpha diversity estimates from single shotgun reads of the metagenomics dataset against the NCBI nr database using the blastX algorithm.

	Taxa		Taxa (without *Hominoidae*)		KEGG		SEED	
samples	Shannon	Simpson	Shannon	Simpson	Shannon	Simpson	Shannon	Simpson
public_buildings	8.44	68.69	8.44	68.67	11.41	1652.82	10.92	1279.98
public_buildings_PMA	8.42	61.75	8.42	61.73	11.38	1616.20	11.01	1276.63
public_houses	8.97	99.16	8.97	99.08	11.58	1814.65	11.00	1255.54
public_houses_PMA	9.00	101.20	9.00	101.12	11.58	1811.69	11.01	1279.79
private_houses	4.98	6.30	4.98	6.30	11.35	1675.57	10.81	1191.86
private_houses_PMA	5.10	6.78	5.10	6.78	11.27	1591.29	10.97	1223.26
gowning_area	7.76	35.81	8.06	42.62	11.86	1947.20	11.02	884.36
ICU	6.27	12.32	7.28	28.89	12.09	1017.25	11.12	1404.91
cleanroom	7.54	21.66	8.42	56.57	11.90	941.92	11.12	1114.67

Table S6A: Summary of applied statistics on the 16S rRNA gene amplicon dataset.

Test	grouping category	metric	significance	details
MRPP	environmental confinement	weighted unifrac	0.001	A: 0.1618, delta 0.4224 of 0.504
	sampling location / surface		0.001	A: 0.1583, delta 0.4242 of 0.504
	environmental confinement	unweighted unifrac	0.001	A: 0.1392, delta 0.6759 of 0.7851
	sampling location		0.001	A: 0.1252, delta 0.6868 of 0.7851
adonis	environmental confinement		0.001	R2: 0.30668
	type of built environment		0.001	R2: 0.51265
	sampling location / surface		0.001	R2: 0.30079
	surface material		0.001	R2: 0.41641
ANOSIM	environmental confinement		0.001	R: 0.633738272592444
	type of built environment		0.001	R: 0.607909664422414
	sampling location / surface		0.001	R: 0.603552220205951
	surface material		0.001	R: 0.380902465808126
two-sided two sample Student's t-tests	environmental confinement	weighted unifrac	distances (maximum to minimum)	controlled vs. moderate controlled 0.6 > moderate controlled vs. uncontrolled 0.59 > controlled vs. Uncontrolled 0.4
	type of built environment			ICU_floor_vs._public_buildings_wp 0.65; public_house_vs._public_buildings_wp 0.36
	sampling location / surface			table_vs._floor 0.6; device_vs._table 0.37
	surface material			metal_polymer_vs._concrete_tiles 0.68; polymer_tiles_vs._stone 0.21
Mantel correlogram	humidity (H)			
	temperature (°C)			
	room volume (vol)			
	surface area (surf)			
	room area (room)			
	surface material (m)			
	geographic latitude (la)			
	geographic longitude (lo)			
	sea level (sl)			
	room height (h)			
BEST (BioEnv)	environmental variables	Bray-Curtis		
	sample		0.9773	
	sample,lo		0.9586	
	sample,la,lo		0.9705	
	sample,la,lo,sl		0.9425	
	sample,vol,la,lo,sl		0.9043	
	sample,vol,H,la,lo,sl		0.8846	
	sample,surf,vol,H,la,lo,sl		0.8659	
	sample,surf,h,vol,H,la,lo,sl		0.8355	
	sample,surf,room,h,vol,H,la,lo,sl		0.8044	
	sample,surf,room,h,vol,H,C,la,lo,sl		0.7738	
	sample,surf,room,h,vol,m,H,C,la,lo,sl		0.7518	

Table S6B: Summary of applied statistics on predicted functions from the 16S rRNA gene amplicon dataset with PICRUSt.

Test	grouping category	metric	significance	details
MRPP	environmental confinement	Bray-Curtis	0.001	A: 0.181, delta 0.06969 of 0.08509
	type of built environment		0.001	A: 0.3074, delta 0.05893 of 0.08509
	sampling location / surface		0.001	A: 0.2373, delta 0.0649 of 0.08509
	surface material		0.001	A: 0.2896, delta 0.06045 of 0.08509
adonis	environmental confinement		0.001	R2: 0.32734
	type of built environment		0.001	R2: 0.55937
	sampling location / surface		0.001	R2: 0.43945
	surface material		0.001	R2: 0.51541
ANOSIM	environmental confinement		0.001	R: 0.482880658436213
	type of built environment		0.001	R: 0.508214886053013
	sampling location / surface		0.001	R: 0.740354908750047
	surface material		0.001	R: 0.395628422683383
two-sided two sample Student's t-tests	environmental confinement		distances (maximum to minimum)	moderate controlled vs. uncontrolled 0.1 > moderate controlled vs. controlled 0.1 > controlled vs. uncontrolled 0.06
	type of built environment			ICU_workplace_vs._private_house 0.14; gowning_area_vs._public_house 0.04
	sampling location / surface			table_vs._floor 0.12; device_vs._table 0.06
	surface material			polymer_tiles_vs._furnished_wood 0.15; polymer_tiles_vs._stone 0.04
Mantel correlogram	room volume (vol)			
	humidity (H)			
	temperature (°C)			
	room area (room)			
	surface area (surf)			
	surface material (m)			
	geographic latitude (la)			
	geographic longitude (lo)			
	sea level (sl)			
	room height (h)			
BEST (BioEnv)	environmental variables			
	sample		0.8918	
	sample, vol		0.8996	
	sample, h, vol		0.8847	
	sample, surf, h, vol		0.8645	
	sample, h, vol, la, lo		0.8448	
	sample, surf, h, vol, la, lo		0.835	
	sample, surf, h, vol, la, lo, sl		0.8123	
	sample, surf, h, vol, H, la, lo, sl		0.7924	
	sample, surf, room, h, vol, H, la, lo, sl		0.7543	
	sample, surf, room, h, vol, m, H, la, lo, sl		0.7262	
	sample, surf, room, h, vol, m, H, C, la, lo, s		0.7024	

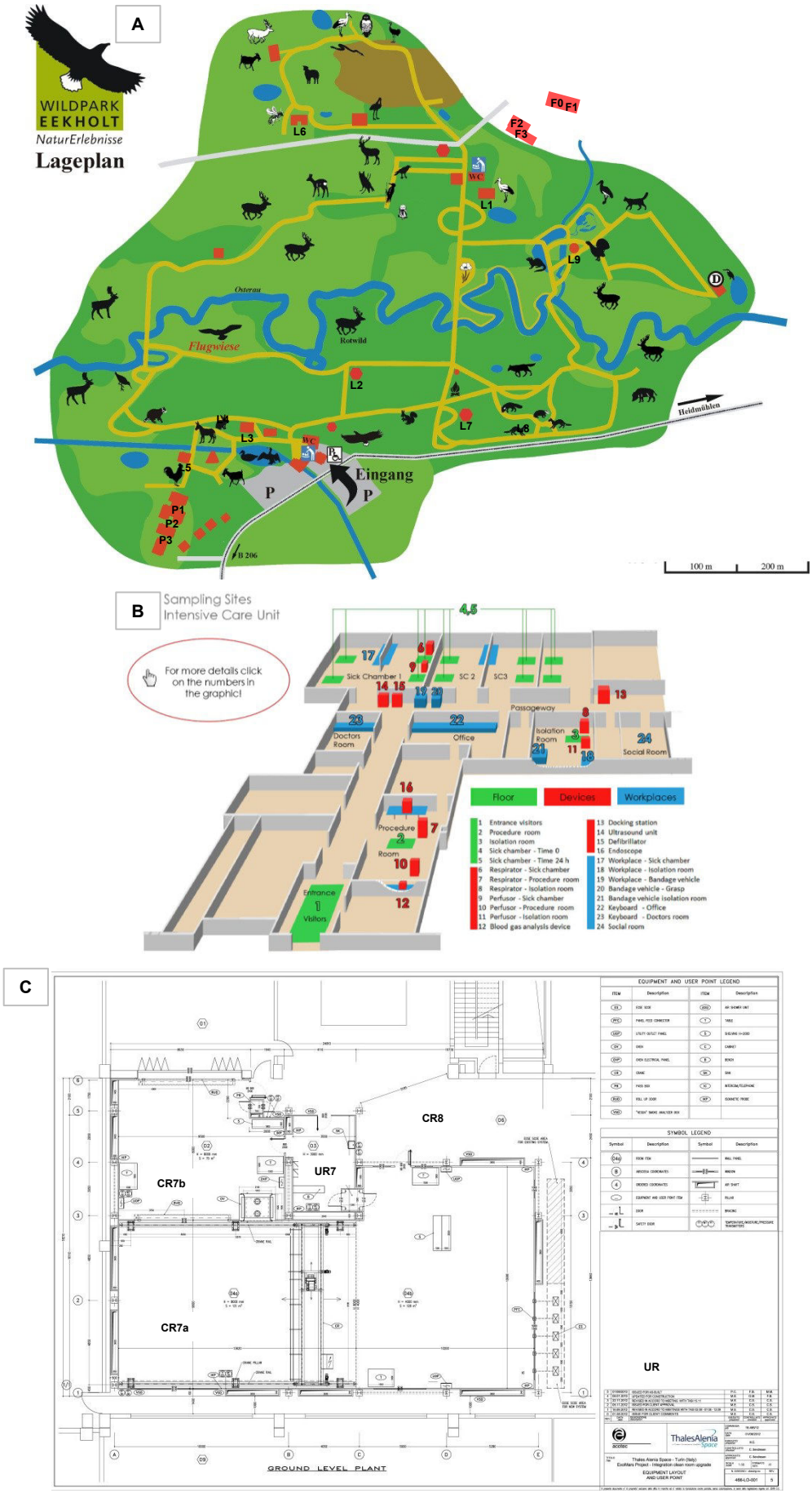


Fig. S1: Sampling maps of **A)** public and private buildings and houses in a wildlife park in Grossenaspe, Germany (top); **B)** the intensive care unit (ICU) at the state hospital Graz (bottom, left); **C)** the Thales Alenia space cleanroom facility in Turin, Italy (bottom, right)

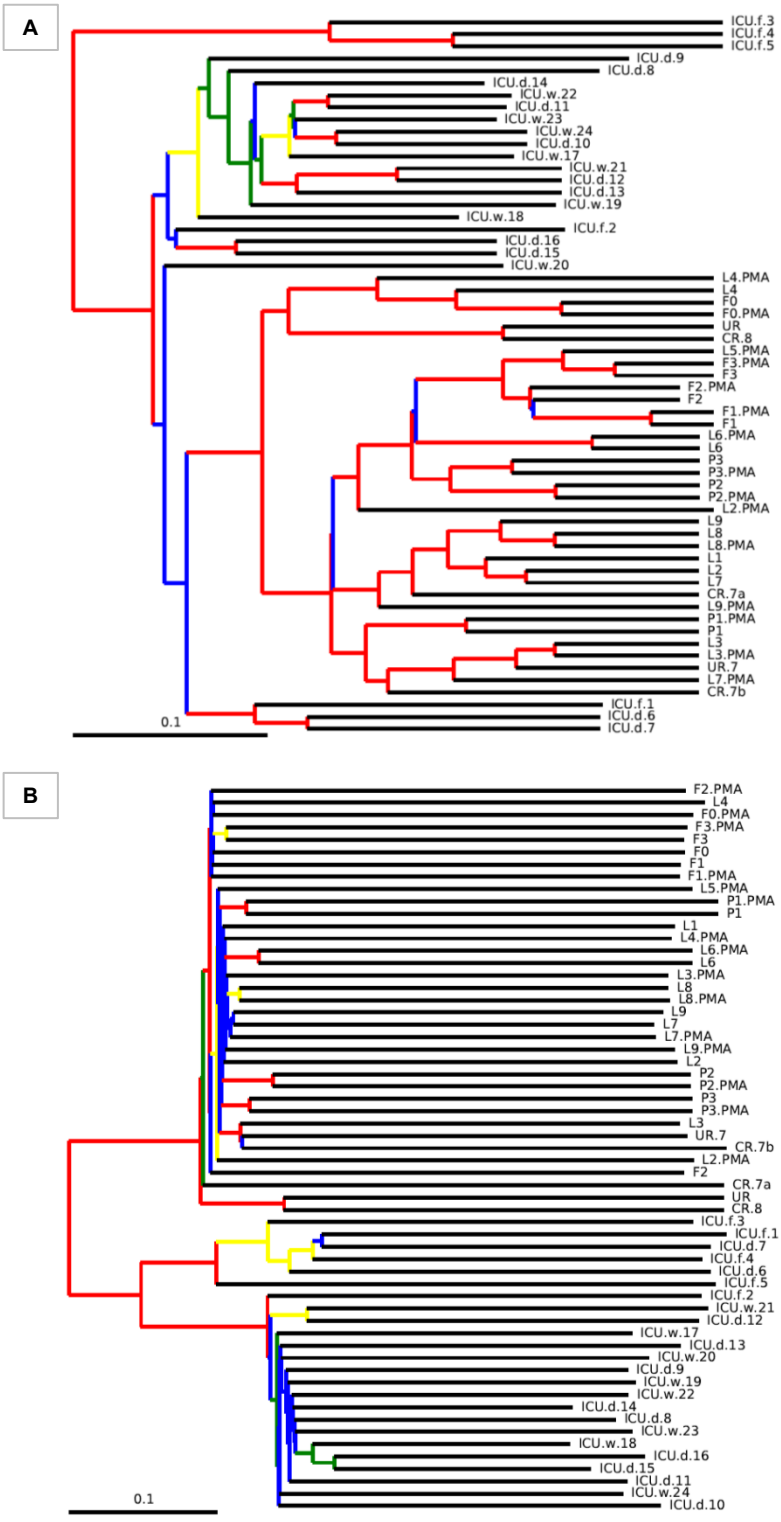


Fig. S2: A) Weighted unifrac, jackknife upgma bootstrapped tree, red 75-100%, yellow 50-75%, green 25-50%, blue < 25%; **B)** unweighted unifrac, jackknife upgma bootstrapped tree, red 75-100%, yellow 50-75%, green 25-50%, blue < 25%

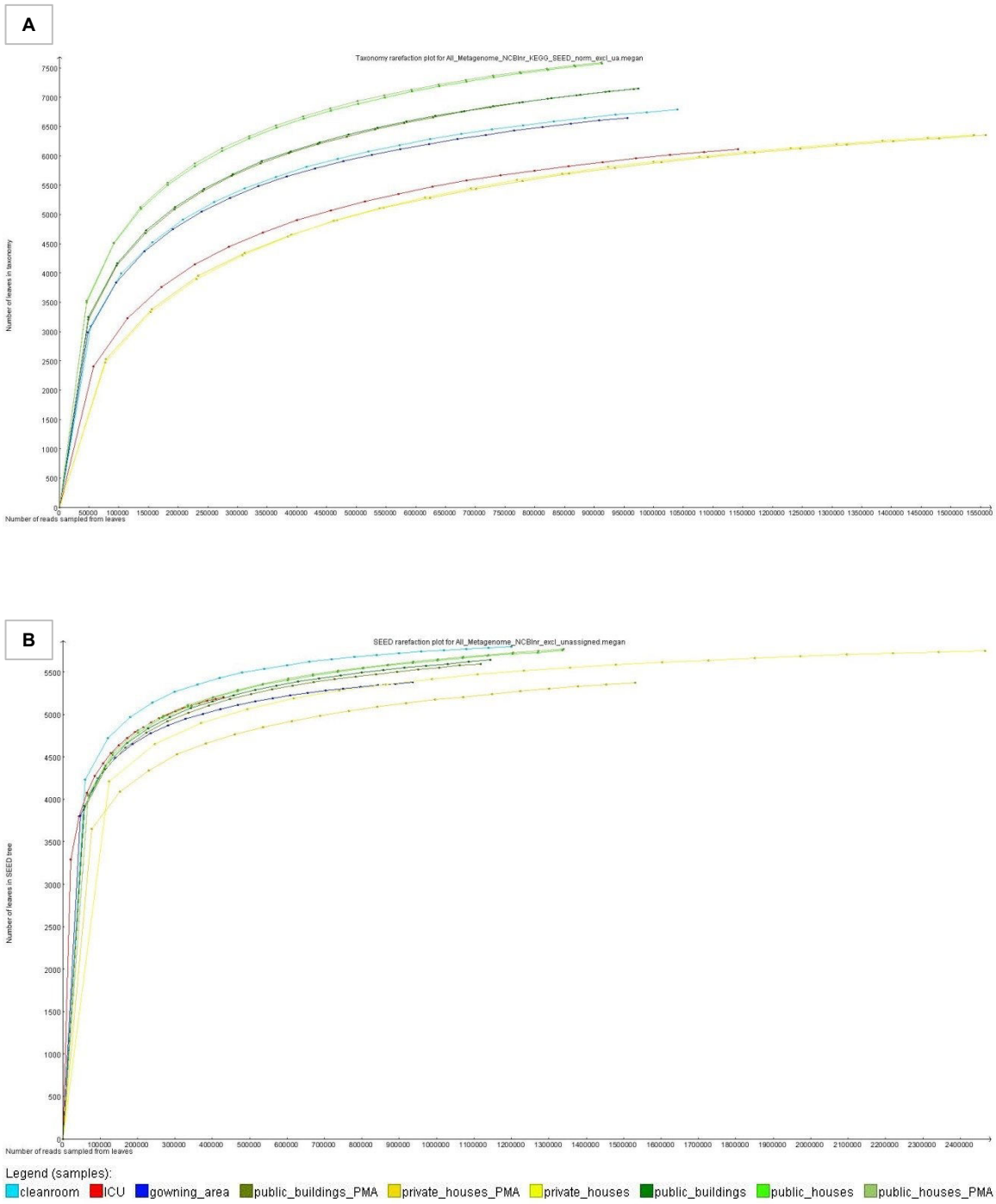


Fig. S3: A) Rarefaction curves of taxa (subspecies level, blastx vs. NCBI nr reads, excluding unassigned, normalized, linear); **B)** Rarefaction curves of all SEED levels (blastx vs. NCBI nr reads, excluding unassigned, normalized, linear).

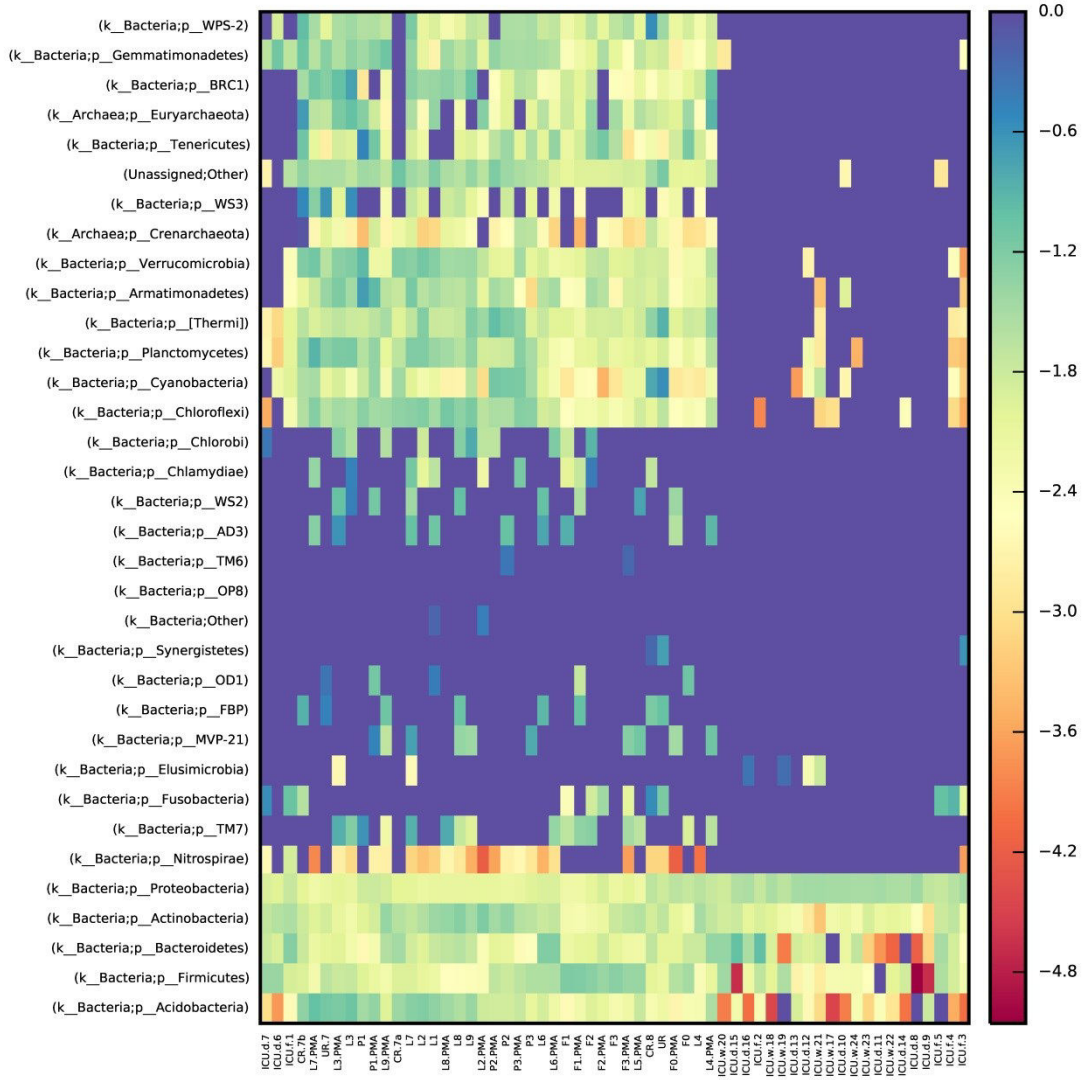


Fig. S4: OTU heatmap on phylum level, based on sampled indoor spaces sorted according to the calculated UPGMA tree, showing the relative abundance of OTUs (from blue – low, via yellow – intermediate, to red – high).

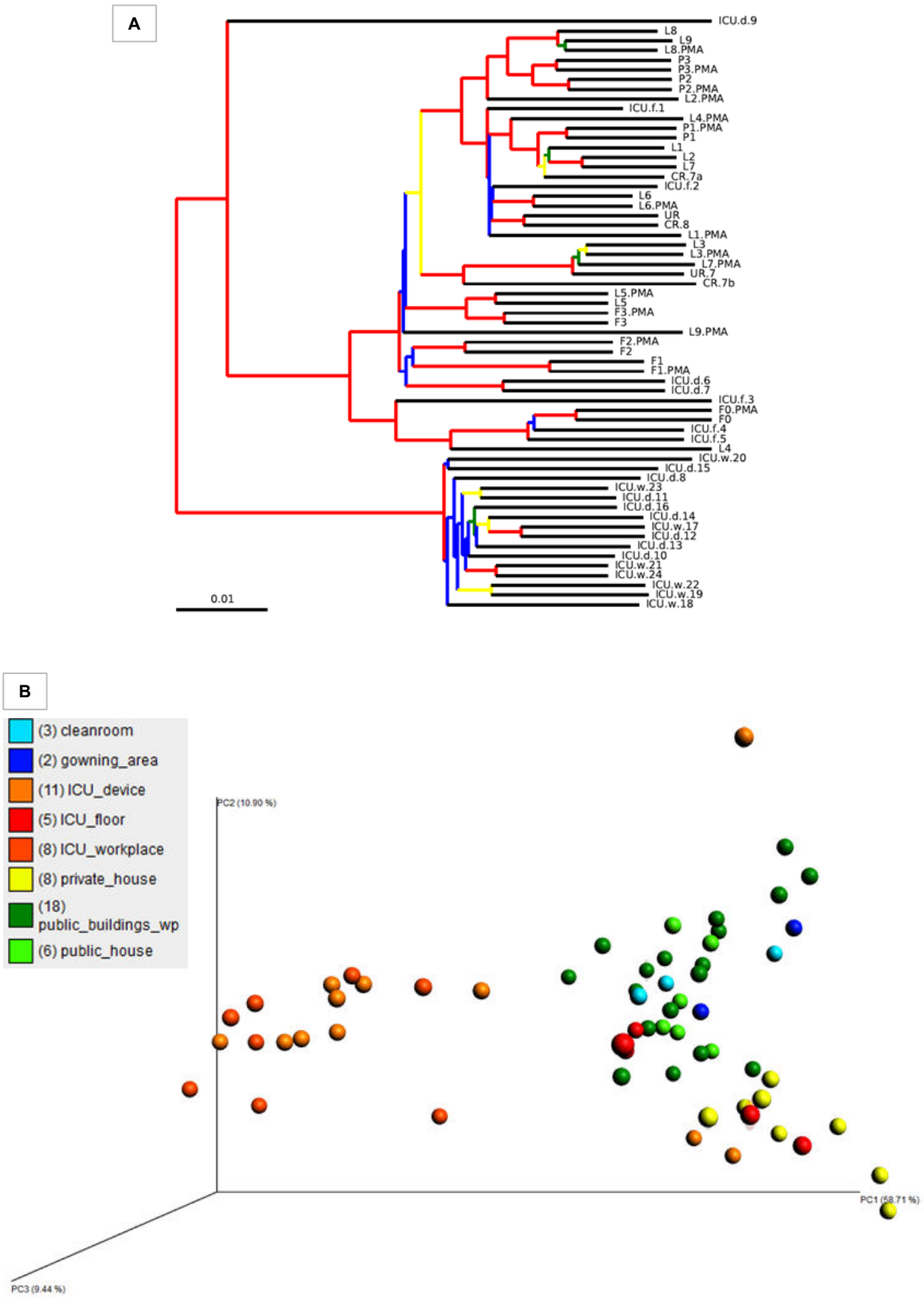


Fig. S5: Analysis on PICRUSt predicted functions from 16S rRNA gene amplicons based on Bray-Curtis distances and KEGG annotations. **A)** Jackknife UPGMA bootstrapped tree based on Bray-Curtis distances, trust indices for calculated branches (red 75-100%, yellow 50-75%, green 25-50%, blue < 25%); **B)** PCoA plot based on Bray-Curtis distances at a depth of 170704 seq/functions;.

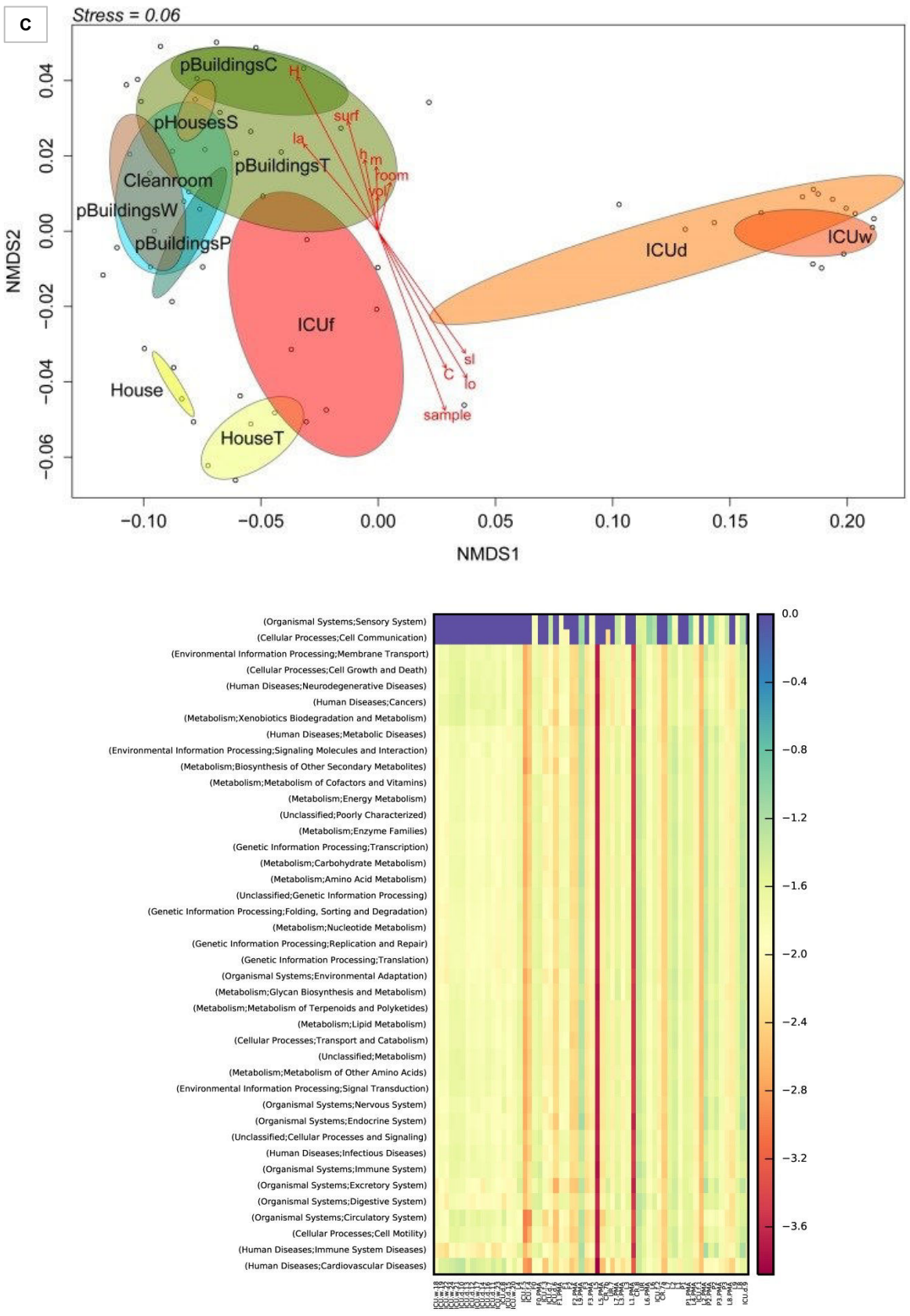
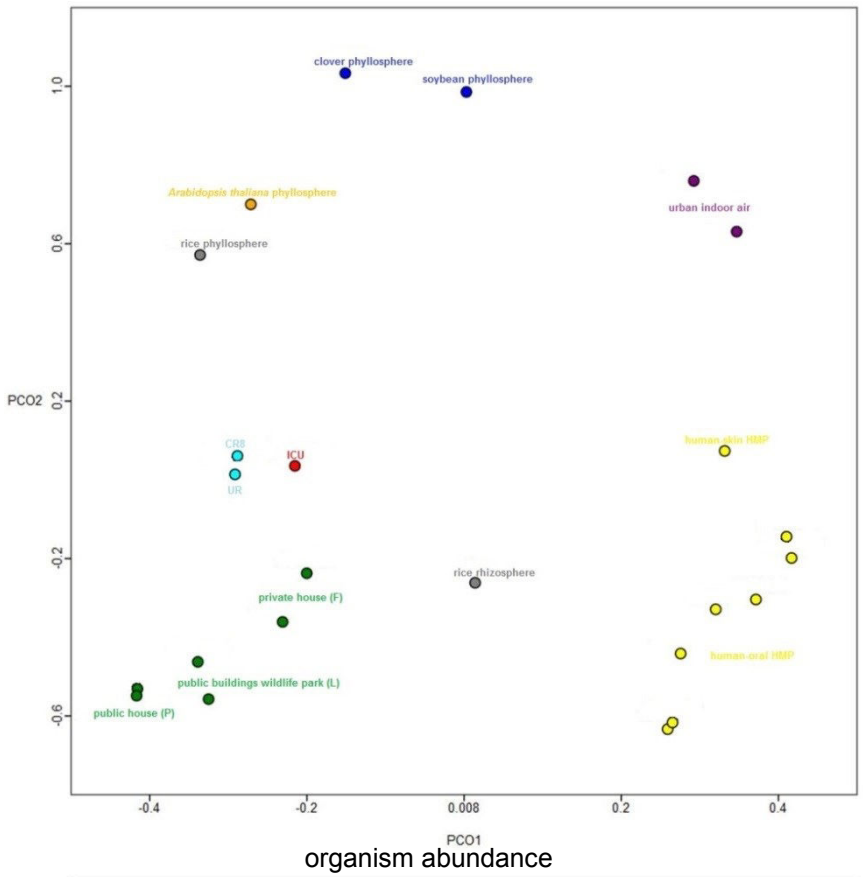
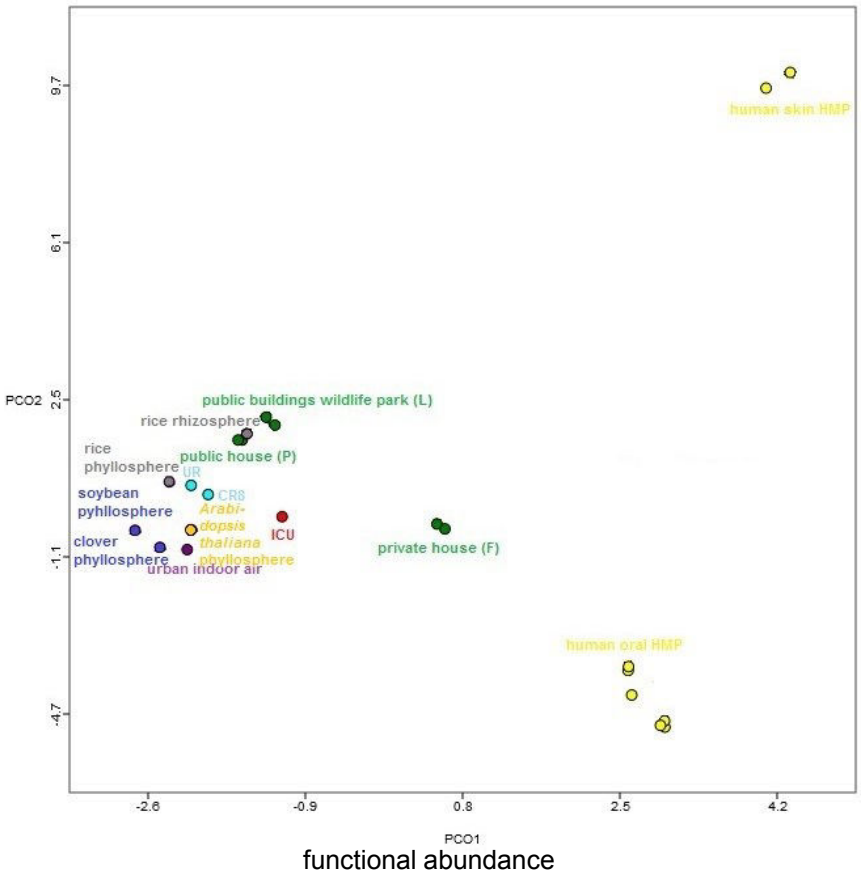


Fig. S5: C) NMDS with superimposed vectors representing Spearman correlations of measured environmental variables (BIO-ENV) based on Eucledian distances. Ellipses for sample groupes were calculated for the following categories: public buildings with concrete (pBuildingsC), tiles (pBuildingsT), polymers (pBuildingsP), wooden floors (pBuildingsW); public houses (pHouses) with samples from sanitary areas (pHousesS); private houses (House) with tiled floors (HouseT); ICU devices (ICUd), floors (ICUf) and workplaces (ICUw), samples of the cleanroom facility (Cleanroom); **D)** Heatmap on KEGG L2 functions, based on sampled indoor spaces sorted according to the calculated UPGMA tree, showing the relative abundance of OTUs (from blue – low, via yellow – intermediate, to red – high).



organism abundance



functional abundance

Fig. S6: Comparative analysis of metagenome samples from different controlled and uncontrolled built environments with metagenome samples from plants, urban indoor air and the human microbiome project on organism and functional abundance levels visualized through MG-RAST.

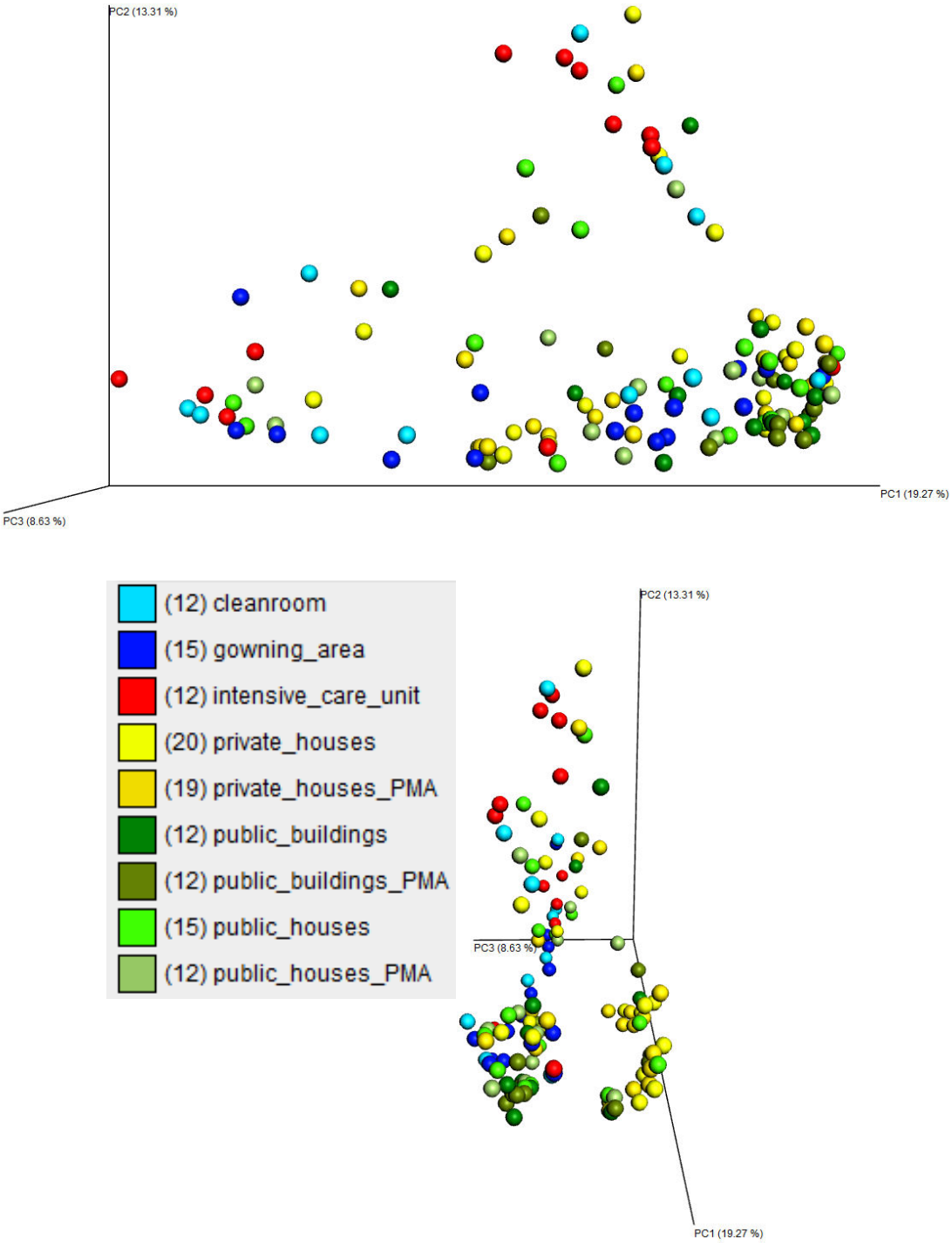


Fig. S7: Bins from the metagenomic dataset annotated with RAST, compared with the RAST sample tool and visualized in QIIME. Top: centered view. Below: view along PC3.