BetterAir Environmental Probiotic Treatment

Introduction

The BioBE (Biology and Built Environment) center in the University of Oregon, conducted fully controlled trials on the Better Air Enviro-Biotics® technology. The University of Oregon performed the testing that generated the data presented in this report.

The project's title was "Testing BetterAir Environmental Probiotic Treatments".

The trials were carried out in April and May of 2018, were hundreds of samples were taken; it has been analyzed from June to December and summarized in a report provided to Better Air in January 2019.
The project has been coordinated by Dr. Jessica L. Green and Dr. Kevin Van Den Wymelenberg, both Co-Directors of the BioBe center. Dr. Green has been a pioneer in the research of the indoor microbiome since 2008 and published numerous papers and publications. Here is a link providing quick review and stage where she presents her work
https://www.ted.com/talks/jessica_green_are_we_filtering_the_wrong_microbes

In these trails, BioBe has been focusing on the microbial viability of the probiotic treatment upon surfaces as well as on the antagonistic effect against indoor pathogenic microbes.

Objectives:

1. Provide BetterAir with data that they can use to estimate microbial viability after probiotic treatment. This includes a provision of data to allow BetterAir to quantify the dosage of live microorganisms delivered to surfaces, and how long those microbes remain alive after treatment.
2. Provide BetterAir with data that they can use to estimate the effect of probiotic treatment on existing microbial community. This includes a provision of data to allow BetterAir to quantify the effect of treatment on common human-associated microbial communities through time with respect to alterations in community structure, diversity, and relative and absolute abundances of target organisms.

Rationale:

1. Demonstration of microbial viability after treatment is necessary to quantify the effects of introducing probiotics into natural microbial communities. Understanding the length of time that these microbes persist in the environment after treatment is discontinued will help to understand and model the effects of episodic treatment regimes.
2. Establishing the effects of probiotic treatment on a human-associated community in tightly controlled experimental conditions is the first step to understanding the effects and potential decay of the treatment in the field.

Experimental samples from the testing were analyzed using different methodologies to achieve our objectives. For the **Viability Analysis** we performed quantitative polymerase chain reaction (qPCR) on treated and control samples from empty sterile plates to track the accumulation and maintenance of living Enviro-Biotics microbes with the use of the BetterAir Biodify device, as well as the viability of those organisms through time after cessation of treatment. We used high throughput sequencing of the bacterial 16S rRNA gene and the ITS region of fungal rRNA operon to produce **Metabarcode Libraries** of treated and control samples of living and sterilized indoor dust, which BetterAir can use to quantify the effect of treatment on community composition and diversity over time. Finally, we performed qPCR on treated and control **Artificial Communities**, composed of *Cladosporium* sp., *Staphylococcus epidermidis*, and *Escherichia coli*, to assess the effect of treatment on known target organisms.

Methods

We used settling plates (100 mm diameter petri dishes) for microbial sampling. Plates were preloaded with either live or sterilized human-associated microbial communities (HAMC), which was obtained from homogenized and filtered vacuum cleaner dust sourced from three distinct occupied Buildings (a multi-family home with a cat, a single family home with a dog, and a damp basement in an academic building). The course of treatment was two weeks of use of the Biodify™ device with standard settings in the ESBL Climate Chamber (interior dimensions = c. 3.7 m long x 2.4 m wide x 2.9 m high; 25.75 m^3) with humidity, air temperature, and air exchange rates held constant throughout the duration of the experiment and within the range typical of residential/commercial buildings. Air exchange was held at the minimum acceptable via ASHRAE standards for occupied office buildings. All incoming air was filtered to MERV 15.
During the treatment period, plates were collected at varying frequencies according to expected trends from each group, with two replicates per group per day.

Sampling days were as follows: Day 0 (Pre-treatment) | (start treatment) | D1, D2, D3, D6, D10, D14 | (stop treatment) |D15, D16, D17, D21. This same sampling protocol was used twice: once for treatment with the Enviro-Biotic mixture, and once for the control, using the device with sterile fluid (no microbes).
Thus a total of 88 samples were collected (11 sampling days x 2 conditions [live vs. sterile dust] x 2 replicates x 2 treatments [Enviro-Biotics vs. control]).

Viability Analysis

We used qPCR and pre-treatment with propidium monoazide (PMA) to compare the amount of total bacteria in the samples to the amount of live bacteria in the empty plate samples that received either Enviro-Biotics® (treatment) or sterile fluid (control). An important assumption is that all of the bacteria observed in the empty plates result from accumulation of the Enviro-Biotic microbes.
Following sample collection, samples were divided into equal portions, with DNA extraction performed in parallel for each: one with PMA pre-treatment to exclude non-viable cells from downstream analysis (living DNA), and one without PMA pre-treatment (total DNA), allowing for viability testing via qPCR. We used DNA standards from Integrated DNA Technologies comprising a 167 bp section of the *E. coli* 16S rRNA region. Original amplification data relies on the assumption that all of the samples amplified at the same efficiency during qPCR. This assumption is typically not true, therefore we used the algorithm LinRegPCR (Ruijter et al., 2009) to apply an individual efficiency correction. All statistical tests and figures were performed using the LinRegPCR-corrected data.

Metabarcode Libraries

We amplified the V3-V4 region of the bacterial 16S rRNA gene and the ITS region of fungal rRNA operon for live and sterilized dust samples that received either Enviro-biotic (treatment) or sterile fluid (control). High-throughput sequencing on the Illumina MiSeq platform was used to generate metabarcode data, which were used to track changes in community structure through time in response to treatment, as compared to changes through time in the control. Due to poor quality of the reverse reads (typical of Illumina paired-end sequencing) and low overlap, we used only the forward reads for downstream analyses. Pre-processing of the raw reads was performed using DADA2. Exact sequence variants output by DADA2 were clustered into operational taxonomic units (OTUs) at 97% similarity using the vsearch de novo method in QIIME2.

Artificial Community

We used qPCR and PMA pre-treatment to determine the amount of live bacteria and *Cladosporium* sp. in the environment. Following sample collection, DNA extraction

was paired with PMA pretreatment to exclude non-viable cells from downstream analysis, allowing only living microbes to be included in our analysis. We used DNA standards from Integrated DNA Technologies, comprising a 167 bp section of the *E. coli* 16S rRNA region, to screen for the abundance of general bacteria over a 24-day period. We used custom-designed primers made from DNA oligonucleotide (Thermo Fisher Custom DNA Oligos) to screen for the presence of *Cladosporium sp.* The custom-designed primers were designed from Sanger sequencing results from extracted DNA that was amplified using ITS primers. In the Artificial Community, both *E. coli* and *S. epidermidis* were quantified by overall 16S bacterial abundance. The LinRegPCR algorithm (Ruijter et al., 2009) was once again used to correct for differences in amplification efficiency between samples.

Statistical Analyses

We used the R statistical computing environment for all pre-processing (except the OTU clustering, which was performed in QIIME2) and analyses, including the packages *dplyr*, *wesanderson*, *ggsignif*, *lme4*, *reshape2*, *dada2*, *DESeq2*, *ggplot2*, *phyloseq*, *vegan*, *ape*, and *plyr*. Linear mixed effects models with likelihood ratio tests and one-way ANOVAs with Tukey's HSD were used to determine significance between treatments.

Results and Discussion

Viability analysis of the Enviro-Biotics® bacteria

The probiotic bacteria accumulated on the surfaces and were found stable also after the cessation of the treatment. There was a significant difference ($F_{3, 83} = 49.5$, $P < 0.001$) between the mean copy number of PMA treated (live) and non-PMA-treated (live + dead) plates. There was also a significant difference in the mean copy number between the control and treated plates of both the total and live conditions ($P < 0.01$) as exhibited in Fig. 1. Tukey's post-hoc comparisons showed an increase in the number of total bacteria 16S copies with Enviro-Biotics treatment ($P < 0.001$).

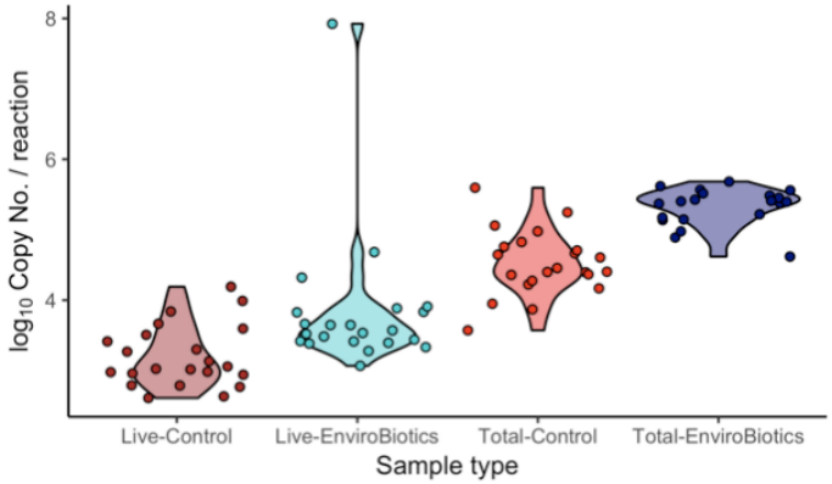


Fig. 1: Comparison of log 10 copy numbers across Enviro-Biotic treated and sterile plates. Live and total counts.

Linear mixed effects modeling showed an increase in total (non-PMA-treated) bacterial load (Fig. 2; $\chi 2$ = 14.85, P < 0.001) through time with the application of Enviro-Biotics treatment. The application of Enviro-Biotics significantly increased the number of live microorganisms on the plates (Fig. 3; $\chi 2$ = 6.62, P =0.01)**.**

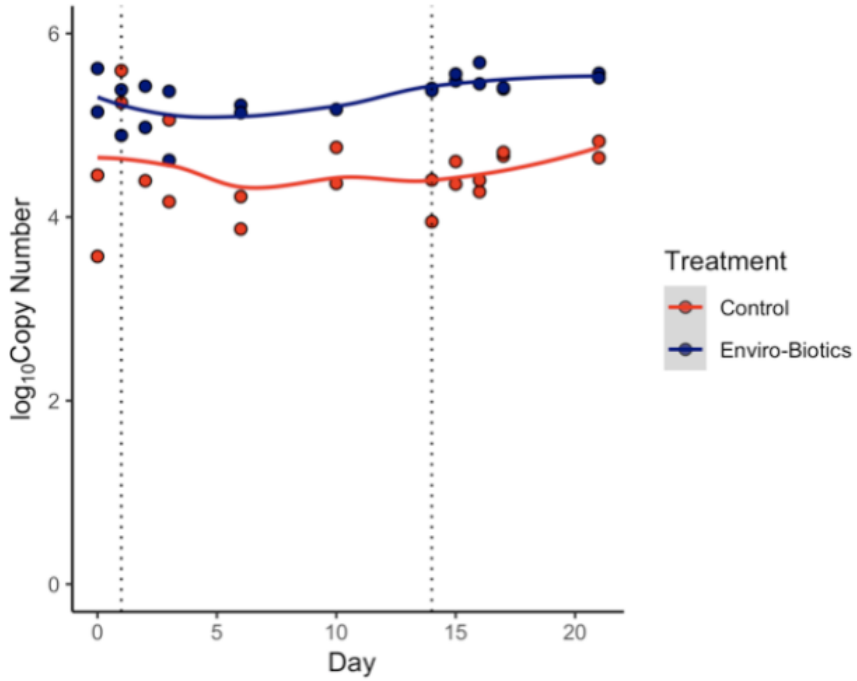


Fig. 2: Comparison of log 10 copy numbers across Enviro-Biotic treated and sterile plates. Counts of total bacteria. The treatment period is defined by the dashed vertical lines.

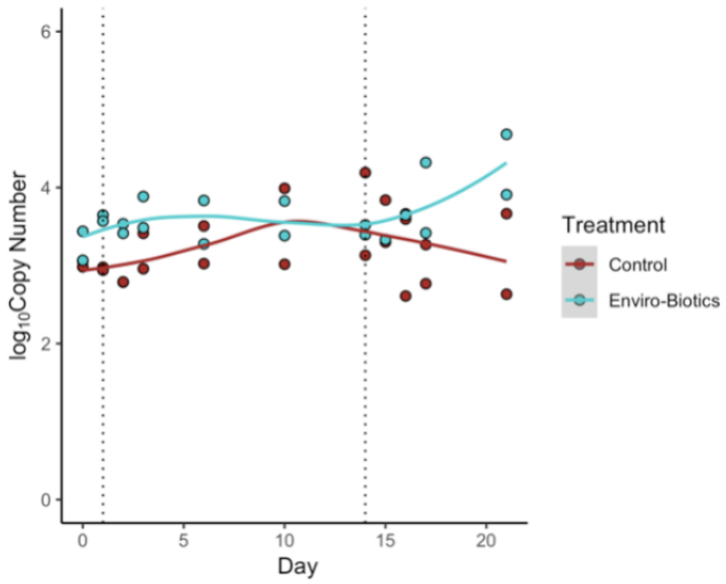


Fig. 3: Comparison of log 10 copy numbers across Enviro-Biotic treated and sterile plates. Counts of live bacteria. The treatment period is defined by the dashed vertical lines.

Following two weeks of application, the concentration of bacteria on empty plates increased due to the Enviro-Biotics® treatment, while the un-treated-control curve showed a decrease in the concentration of bacteria.

This data indicates that the probiotic bacteria are stable on indoor surfaces and that continuous application is due to boost their presence and performance.

Metabarcode Library Analysis

16S Metabarcode library and Bacterial community structure

In this study, we observed a total of 4,915,233 sequence reads across all experimental samples. The number of reads per sample ranged from 12,347 to 92,048, although 2 samples were below our threshold of 10,000 reads and were removed from downstream analyses. The highest number of read counts (127,232) was observed in the used Enviro-Biotics® spritzer. A total of 3,150 different taxa were observed, representing 38 unique phyla.

The top 25 most abundant genera in treated and control samples across both sterile and live dust is depicted in Fig. 4. *Bacillus* genus was observed in the top most abundant genera as can be seen in Fig. 4A. Further analysis of specifically *Bacillus*, the component of Enviro-Biotics® was carried out.

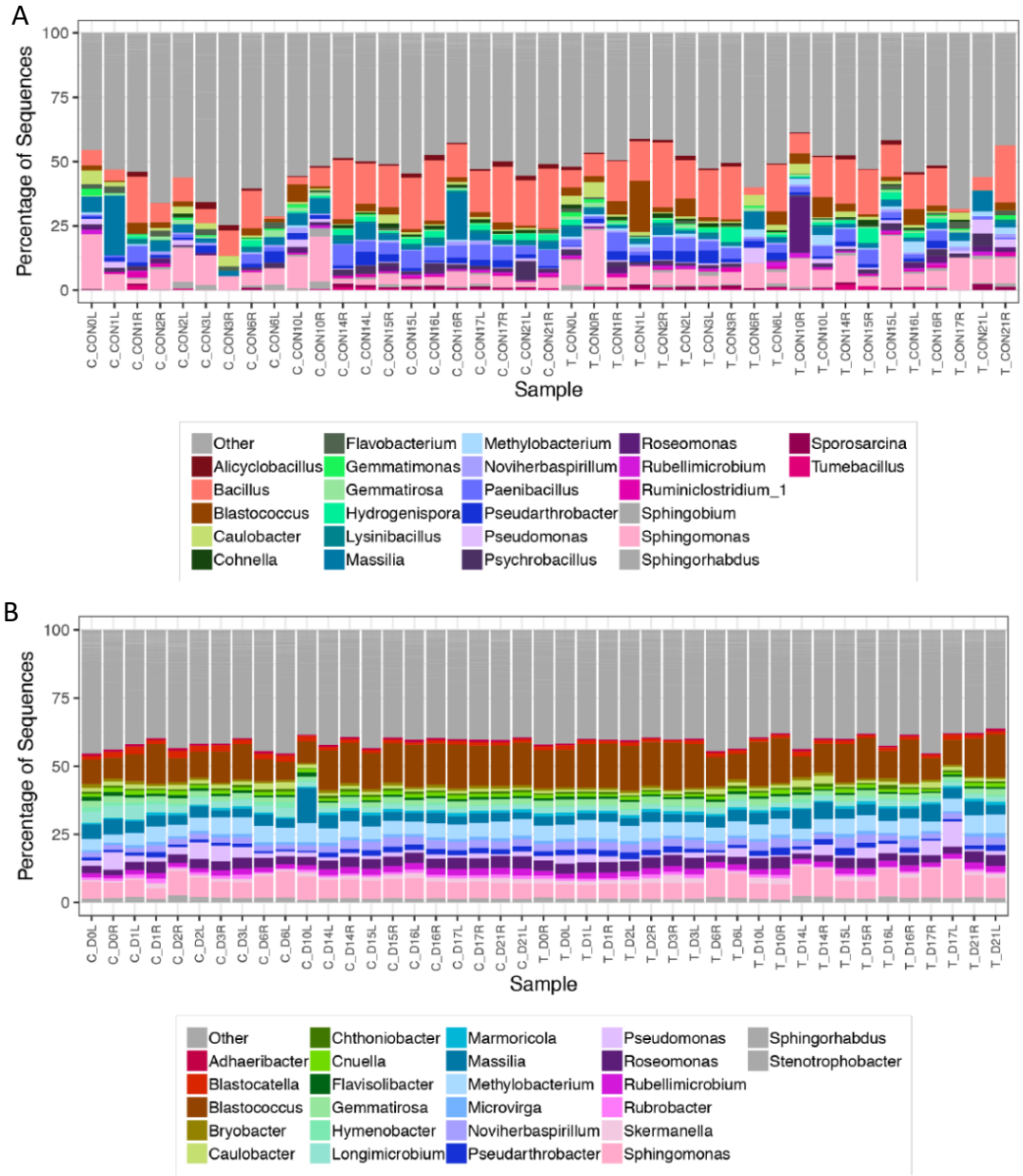


Fig. 4: Top 25 most abundant fungal genera observed in sterile (A) and live (B) dust.

The relative abundance of the top taxon (*Bacillus subtilis*) found in the Enviro-Biotics® solution was specifically visualized to assess whether its abundance changed over the course of the experiment.

In the sterile plates the relative abundance of *B. subtilis* fluctuated between 0–2% for the control samples and increased from 0 to over 4% for the treated samples, showing an increasing trendline overtime as presented in Fig. 5.

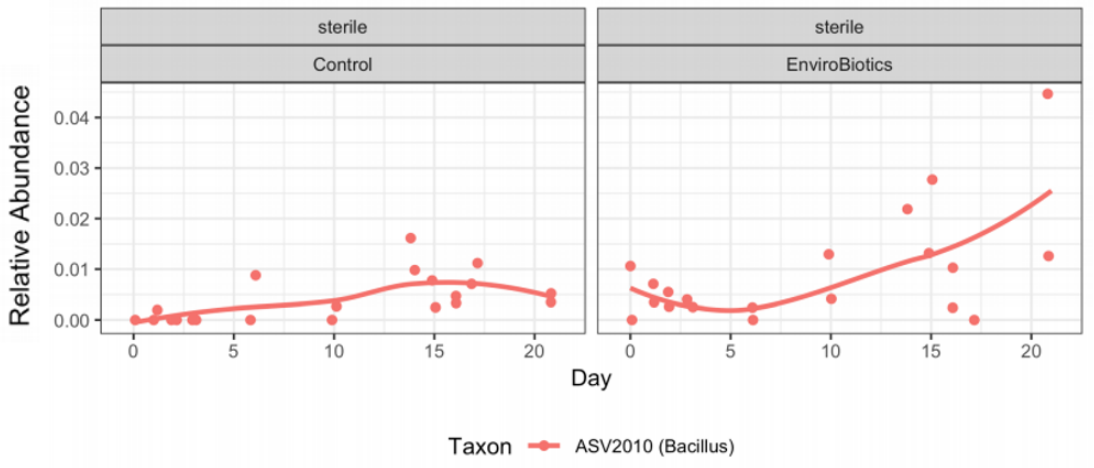


Fig. 5: Change in relative abundance over time for the top taxon found in sterile dust.

These results support the former findings indicating that the Enviro-Biotic bacteria establish in new environments even after a short application regime. They keep propagating also after the termination of their application.

ITS Metabarcode library and Fungal community structure

In this study, we observed a total of 3,779,060 sequence reads across all experimental samples. The number of reads per sample ranged from 105 to 133,869, although 11 samples were below our threshold of 100 reads and were removed from downstream analyses. A total of 4,037 different taxa were observed, representing 13 unique phyla. We noted that the number of reads observed per sample were orders of magnitude higher for live dust (median reads/sample = 94,010) than for sterile dust (median reads/sample = 386).

For alpha diversity, species richness for treatment vs. control in both sterile and live dust (Fig. 6) was calculated using the Chao1 richness estimator. Control samples had slightly higher alpha diversity than treated samples, which was significant for live dust conditions (Welch's t-test; $t = 3$, $p = 0.001$), but not for sterile (Welch's t-test; $t = 2$, $p = 0.1$). Live dust, on the other hand, had significantly higher alpha diversity than sterile dust for both treatment (Welch's t-test; $t = 8$, $p < 0.001$) and control samples (Welch's t-test; $t = 10$, $p < 0.001$). A pattern of decreasing richness over time was observed in the treated live dust samples (Fig. 7), which may be indicative of inhibitory effects of the Enviro-Biotics® treatment.

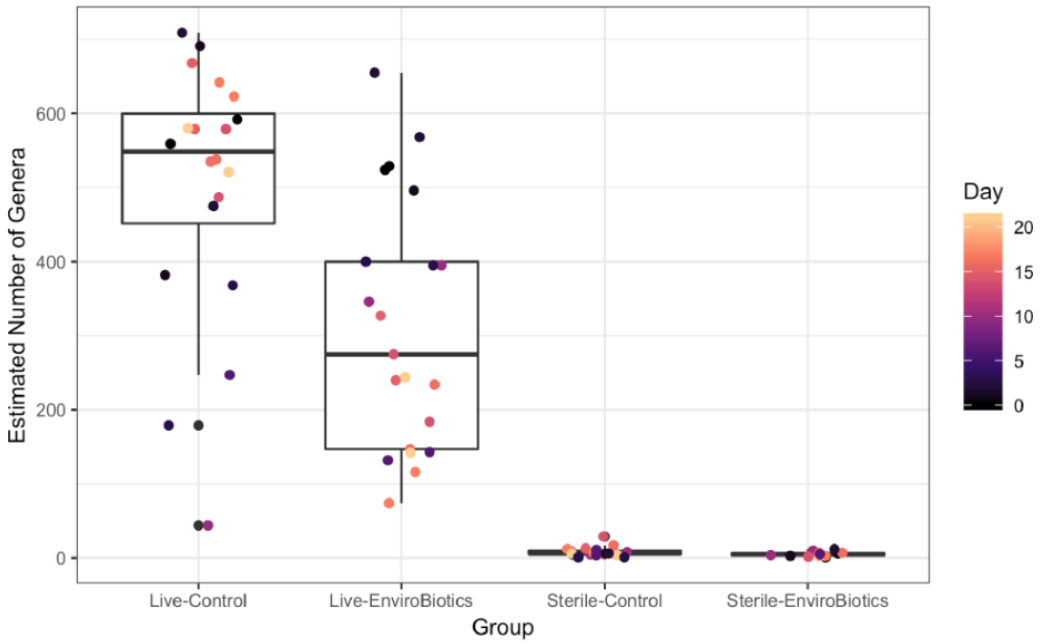


Fig. 6: Chao1 richness estimates for treated and control samples across live and sterile dust.

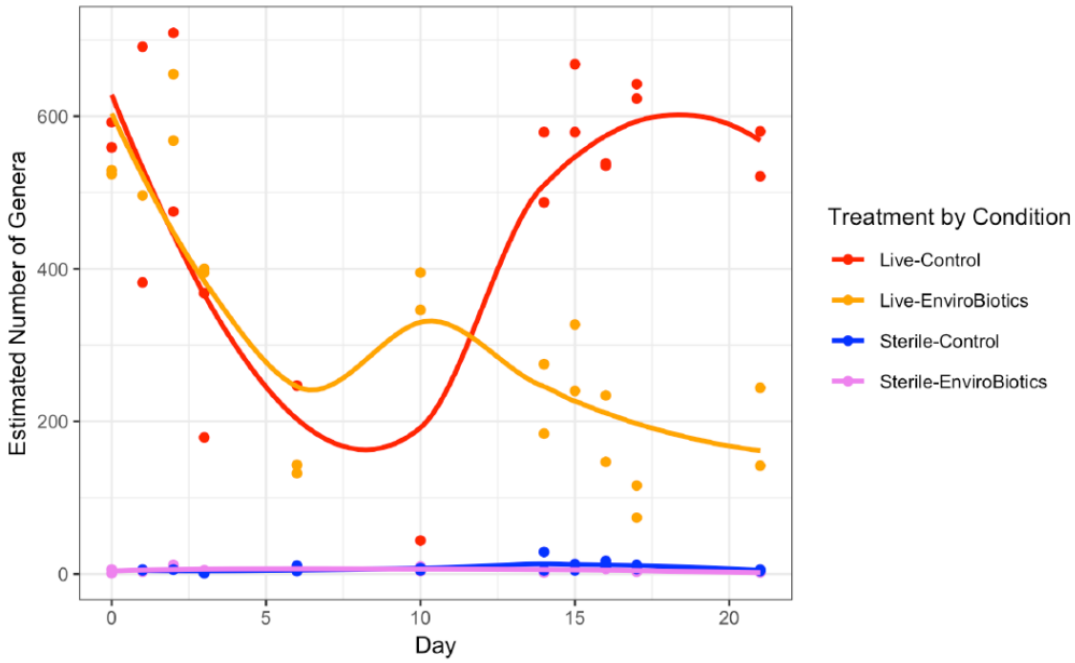
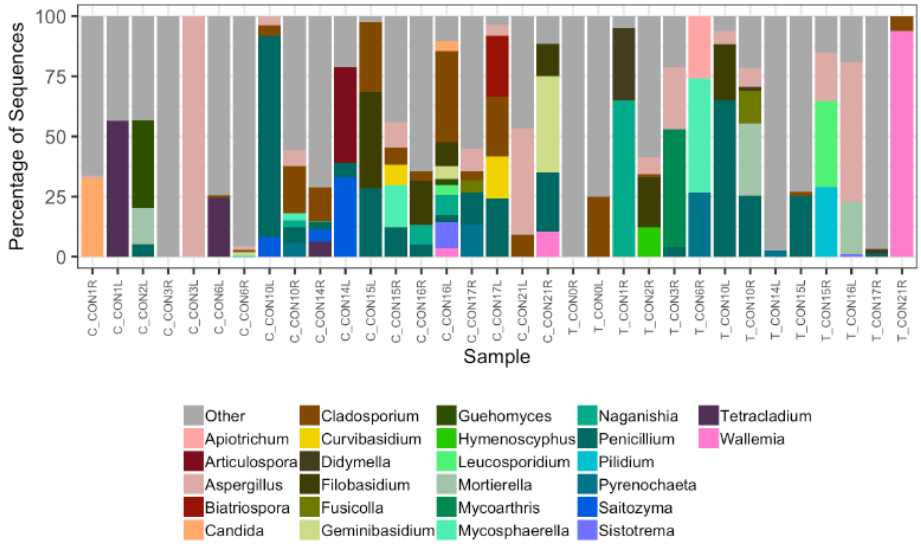


Fig. 7: Estimated Richness over time by experimental group.

The top 25 most abundant genera of fungi in treated and control samples across both sterile and live dust were compared (Fig. 8).

A



B

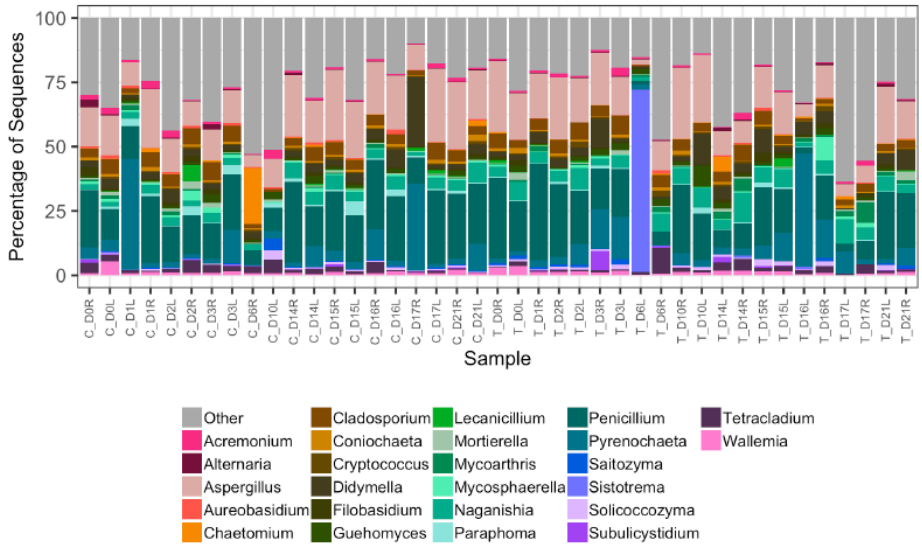


Fig. 8: Top 25 most abundant fungal genera observed in sterile (a) and live (b) dust.

Efficacy against pathogens according to artificial community analysis

The probiotic treatment exhibited a clear antagonistic effect against three typical indoor microbial pathogens:

Escherichia coli – a pathogenic bacteria causing a wide range of human related infections.

Staphylococcus epidermidis - a Gram-positive bacterium that infects patience with compromised immune systems, people with catheters and is a frequent contaminant of specimens sent to the diagnostic laboratory.

Cladosporium sp. - an indoor pathogenic mold. Its airborne spores are significant allergens that severely affect asthmatics.

The mean copy numbers of bacteria and fungi in treated plates were significantly lower than in control plates (Fig. 9; bacteria: $F1, 42 = 4.272$, $P = 0.05$; fungi: $F1, 42 = 10.66$, $P < 0.01$).

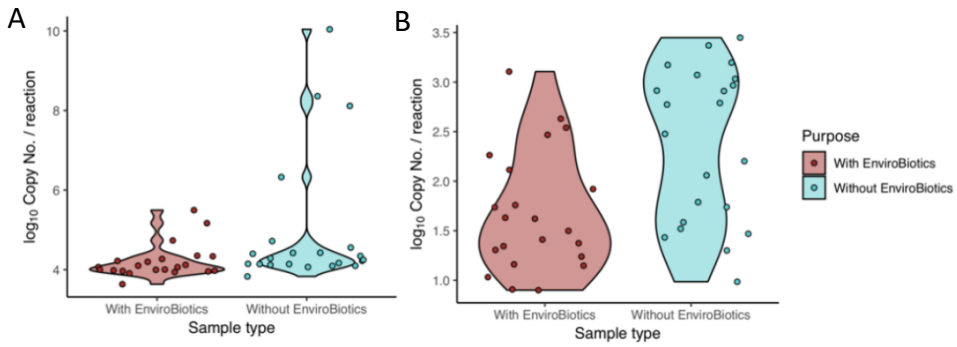


Fig. 9: Comparison of $\log_{10}$ copy numbers across Enviro-Biotic treated and control plates of (A) 16S bacteria *(E. Coli* and *S. epidermidis*) and (B) *Cladosporium sp*.

The inhibition effect over *E. Coli* and *S. epidermidis* is depicted in Fig. 10. Continuous application for 14 days suppressed the pathogenic bacteria counts in comparison to the control.

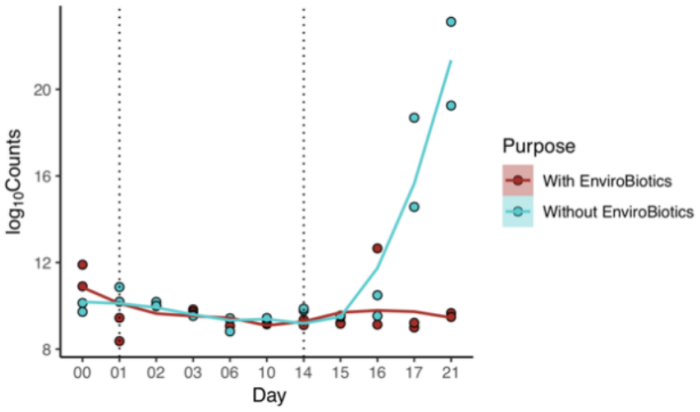


Fig. 10: Counts of live *E. coli* and *S. epidermidis* over time. Enviro-Biotics® treated plates received treatment from day 1 to day 14. The treatment period is defined by the dashed vertical lines.

Following the 14 days treatment regime, the *E. coli* and *S. epidermidis* counts in the un-treated-control increased dramatically, while their counts in the treated samples were kept low due to the probiotic inhibition effect.

The antagonistic effect against *Cladosporium sp*. was even more impressive as can be seen in Fig. 11: *Cladosporium sp.* counts increased significantly more over time in control versus treated samples (Fig. 11; $\chi 2$ = 12.3, P < 0.001).

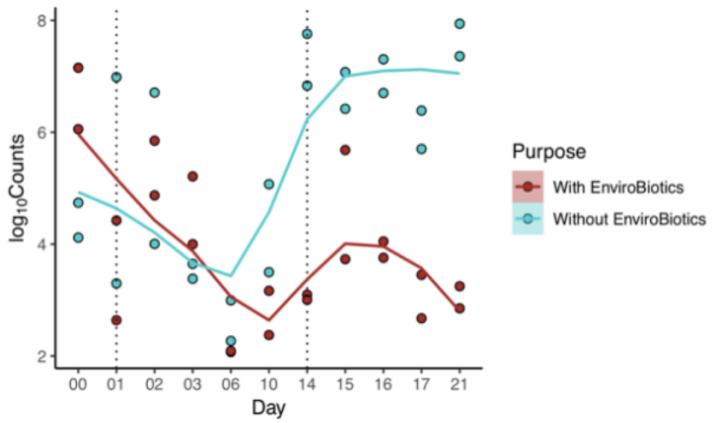


Fig. 11: Counts of live *Cladosporium sp.* over time. Enviro-Biotics® treated plates received treatment from day 1 to day 14. The treatment period is defined by the dashed vertical lines.

Therefore, it can be concluded that from the 8th day onwards, a significant antagonistic effect was demonstrated over the *Cladosporium sp.* pathogenic fungi.

Conclusion

We conclude that for the empty plate viability analysis, the Enviro-Biotics® treatment adds 16S DNA to the environment and increases the numbers of viable cells.

It can be concluded from the metabarcode library analysis that in the sterile plates the relative abundance of *B. subtilis* showed an increasing trendline overtime.

According to the artificial community analysis, we conclude that the Enviro-Biotics® treatment exhibited a clear antagonistic effect against three typical indoor microbial pathogens: *Escherichia coli*, *Staphylococcus epidermidis* and *Cladosporium sp.* It is noted that these species typical indoor microbial pathogens with a potential to be pathogenic and harmful to people, able to cause a wide range of human related infections or indoor acquired illnesses.

To our knowledge, this is the first experiment showing viability and effect of probiotic bacteria on real home dust with its natural microbes, as well as sterilized dust with artificial community, all done in a controlled Climate Chamber, with dimensions as big as a regular big room with humidity, air temperature, and air exchange rates held constant throughout the duration of the experiment and within the range typical of residential/commercial buildings.

The conclusion of the project's final report that was issued on February 20th, 2019, is summarized by the following words:

"There were significantly lower numbers of *Cladosporium sp.* in treated versus control samples, as well as a decrease over time in *Cladosporium* numbers during treatment with Enviro-Biotics. This evidence suggests that Enviro-Biotics treatment could play a role in decreasing *Cladosporium sp.,* and potentially other molds and mildews within the environment."

Bibliography

Ruijter, J. M., Ramakers, C., Hoogaars, W. M. H., Karlen, Y., Bakker, O., Van den Hoff, M. J. B., &
Moorman, A. F. M. (2009). Amplification efficiency: linking baseline and bias in the analysis of
quantitative PCR data. Nucleic acids research, 37(6), e45-e45.