Clinically Relevant Pathogens on Surfaces Display Differences in Survival and Transcriptomic Response in Relation to Probiotic and Traditional Cleaning Strategies

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Abstract

Prolonged survival of clinically relevant pathogens on inanimate surfaces represents a major concern in healthcare facilities. Contaminated surfaces can serve as reservoirs of potential pathogens and greatly hinder the prevention of healthcare-associated infections. Probiotic cleaning using environmental microorganisms to promote inter-species competition has been proposed as an alternative to traditional chemical-based cleaning using antimicrobials. Probiotic cleaning seeks to take advantage of ecological principles such as competitive exclusion and utilize benign microorganisms to inhibit viable pathogens on indoor surfaces. However, limited mechanistic study has yielded direct evidence that enables the scientific community to understand the stress response, or microbe-microbe interactions between healthcare-associated pathogens and probiotic bacteria. Therefore, to bridge this knowledge gap, we combined transcriptomics and traditional microbiology techniques to investigate the differential impact of chemical-based and probiotic surface cleaners on the survival of *Acinetobacter baumannii* and *Klebsiella pneumoniae*, two clinically important pathogens. Although probiotic *Bacillus* included

in a commercialized All-Purpose Probiotic Cleaner persisted on surfaces for an extended period of time, surfaces contaminated with *A. baumannii* cleaned using chemical-based detergent with and without probiotic *Bacillus* showed no statistical difference in the viable colony forming units (CFUs) of *A. baumannii*. Similarly, for *Klebsiella pneumoniae*, there were negligible statistical differences in CFUs between probiotic and detergent cleaning scenarios. The transcriptome of *A. baumannii* with and without probiotic addition shared a high degree of similarity in overall gene expression, while the transcriptome of *K. pneumoniae* with and without probiotic addition differed in overall gene expression. Together, these results highlight the need to fully understand the underlying biological and ecological mechanisms for different pathogens and practical implications of probiotic indoor cleaning.

Keywords: probiotic cleaner; *Acinetobacter baumannii*; *Klebsiella pneumoniae*; transcriptomic response; indoor surface cleaning; infection prevention

1. Introduction

Acinetobacter baumannii and *Klebsiella pneumoniae* have been recognized as a major source of healthcare-associated infections associated with high morbidity and mortality rates due to their resistance to many antibiotics including carbapenems[1] [2]. *Acinetobacter* and *Klebsiella* species are capable of contaminating and persisting on inanimate surfaces for up to several months [3], significantly increasing the risk of acquisition for hospitalized patients who are immunocompromised. Given the concern with transmission of infectious microorganisms including *Acinetobacter* and *Klebsiella*, many cleaning products contain added antimicrobials as disinfectants. However, questions remain about the extent to which chemicals, particularly antimicrobial products, reduce nosocomial infections and whether misuse of these products leads to antimicrobial resistance[4–6]. Several recent studies have demonstrated that cleaning using antimicrobials can effectively reduce microbial load on contaminated surfaces in health care facilities[7]. However, their effects on preventing disease transmission and reducing healthcare-acquired infections remain limited[8]. In addition to concerns about antimicrobial resistance, exposure to cleaning and disinfection agents can cause both acute and chronic health impacts. Healthcare workers with pre-existing respiratory illness, such as asthma, are particularly vulnerable[9]. As a result, an alternative strategy using microbiome manipulation draws increasing attention, seeking to promote inter-species competition between pathogens and probiotic microorganisms, namely *Bacillus*[10–14] , rather than using chemicals to combat pathogens in the built environment.

Falagas and Makris first proposed to use environmental probiotic microorganisms and/or biosurfactants to suppress the colonization of nosocomial pathogens and subsequently reduce nosocomial infections in healthcare facilities in 2009[13]. Since then, much work on the probiotic cleaning potential of *Bacillus* has been performed[10–12,14]. *Bacillus* is a genus of bacteria commonly found indoors[15]. Species of *Bacillus* are generally recognized as safe[16] , with a few exceptions (e.g., *Bacillus anthracis*). In addition to its prevailing presence, *Bacillus* bacteria are capable of forming spores[17] , which can remain viable for an extended period of time, making them ideal for storing in detergents.

Probiotic cleaning seeks to take advantage of competitive exclusion and other ecological principles to inhibit viable pathogens on surfaces[4,18], but there is limited direct evidence documenting these phenomena, along with stress response, or microbe-microbe interactions between probiotic and healthcare-associated pathogens. One major challenge is the technical limitations of many techniques, e.g., transcriptomic sequencing of low biomass samples collected from the built environment often suffers from a low signal-to-noise ratio. Therefore, to obtain a fundamental mechanistic understanding of indoor cleaning strategies and their impacts on microbial survival and interactions, we utilized microcosm chambers to simulate a simplified built environment with greater than usual microbial load. We seek to quantify the effects of probiotic cleaning on viability reduction of healthcare-associated pathogens (e.g., *A. baumannii* and *K. pneumoniae*) and evaluate inter-species interactions between *Bacillus* contained in a probiotic surface cleaner and healthcare-associated pathogens under ambient conditions and elevated temperature and humidity. Cleaning and probiotic products represent a multi-billion-dollar market and a critical tool in our arsenal against microbial infections. Being able to fully

understand microbe-chemical and microbe-microbe interactions will not only help determine the most successful contexts for probiotic cleaning, but also lead to better future product formulation.

2. *Materials and Method*

2.1. Microcosm set-up

A commercially available All-Purpose Probiotic Cleaner (APPC) was obtained from Graz, Austria. Clinical isolates of *Acinetobacter baumannii* (ABBL18) and *Klebsiella pneumoniae* (CRE231) were provided by Dr. Alan Hauser, Northwestern University Feinberg School of Medicine. To simulate a typical indoor surface environment, 24G stainless steel sheets were cut into 2"x2" squares. *A. baumannii* (ABBL18) and *K. pneumonia*e (CRE231) isolates were grown in 40 mL tryptic soy broth (TSB) for 23 hours at 30°C with continuous shaking to its mid log phase. The APPC was filtered through a 0.22 μm syringe filter to create an All-Purpose Cleaner (APC) without probiotic materials as a control. Vegetative *Bacillus spp*. (VB) culture was prepared by mixing 0.1% APPC with TSB. This culture was incubated at 25°C for 23 hours with continuous shaking. Cells (*Bacillus spp*., *A. baumannii and K. pneumoniae*) were harvested and washed in phosphate-buffered saline (PBS) before inoculation.

A total of six testing scenarios for each pathogen (twelve groups in total) are specified as follows:

Table 1 Testing scenarios for each pathogen (*A. baumannii* and *K. pneumoniae)* consisting of four cleaning conditions, and two temperature and humidity conditions.

Cleaning Scenarios	Temperature and humidity conditions	
	25°C and 20% RH	37°C and 90% RH
Pathogen	Pathogen (A)	Pathogen (W)
Pathogen + APC	Pathogen + APC (A)	NA
Pathogen + APPC	Pathogen + APPC (A)	Pathogen + APPC (W)
Pathogen + VB	Pathogen + VB (A)	NA

Each 2"x2" stainless steel surface was first inoculated with 50 μL *A. baumannii* (corresponding to 8.5×10^7 CFU) or 50 μL *K. pneumoniae* (corresponding to 1.0×10^8 CFU). 50 μL sterilized PBS buffer, APC, APPC, or vegetative *Bacillus spp*. inoculum was added to create no-cleaning control samples (ABBL18 or CRE231), chemical cleaning samples (ABBL18 + APC or CRE231 + APC), probiotic cleaning samples (ABBL18 + APPC or CRE231 + APPC), or vegetative cleaning samples (ABBL18 + VB or CRE231 + VB), respectively. Following the introduction of bacterial inoculum, all surfaces were transferred into autoclaved mason jars under two treatment conditions: 1) ambient temperature (25°C) and relative humidity (20%), and 2) elevated temperature (37°C) and relative humidity (>90%) with 10 mL of autoclaved milliQ water. All microcosm chambers were stored in incubators maintained at 25°C or 37°C. Swab samples were collected at three specific timepoints: 3 hours, 24 hours, and 72 hours following the inoculation and cleaning. Triplicate samples were prepared for each combination of cleaning, timepoint, and incubation conditions (Figure 1).

2.2. *Swab sample collection*

Swab samples were collected through a combination of dry and wet swabbing as performed previously[19]. Each surface was first dry swabbed from left to right for seven times and the swab was swirled into an aliquoted PBS buffer or RNAprotect Bacteria Reagent (Qiagen), for CFU enumeration and RNA extraction, respectively. Surfaces were then wet swabbed for a total of 20 s, including 5 seconds of rinse and rewet. Finally, the swabs were left in 15 mL conical tubes and vortexed for 10 seconds. 100 μL swab suspension along with its dilutions were spread onto tryptic soy agar (TSA) and CHROM *Acinetobacter baumannii* selective agar and on MacConkey agar as *Klebsiella pneumoniae* selective media[20] and incubated at 37°C. Numbers of colony forming units (CFUs) were counted after three days.

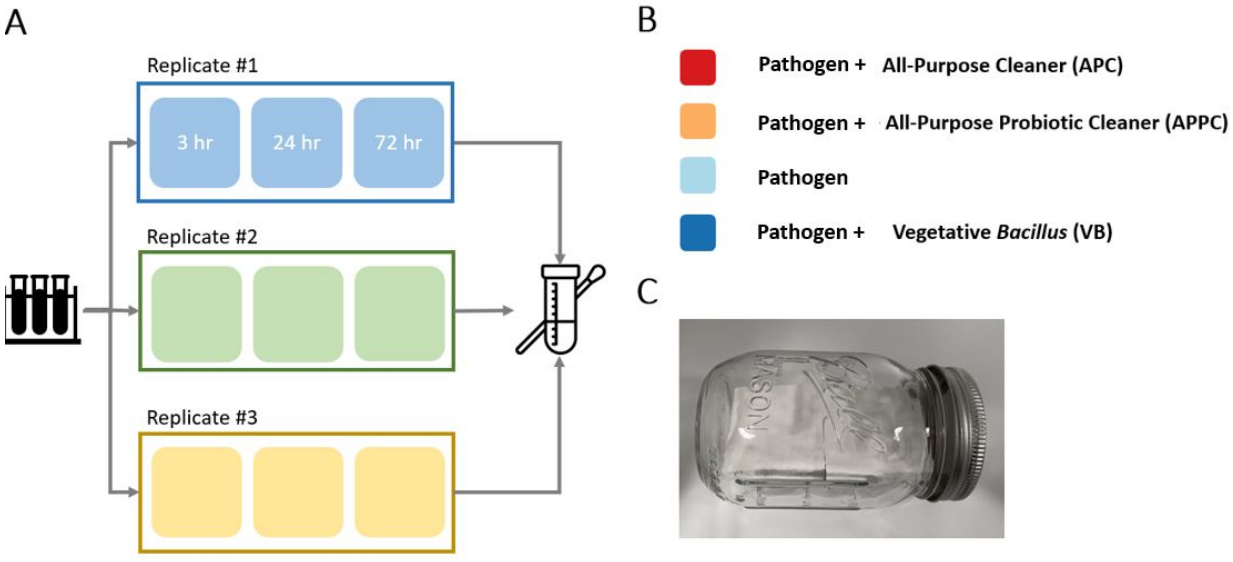


Figure 1. Microcosm experimental design (A) with four types of inoculums (B) containing pathogen (*A. baumannii* or *K. pneumoniae*) and *Bacillus spp.* and sealed sterile microcosm chamber maintained under ambient temperature and relative humidity (C).

The elevated temperature and humidity condition was maintained by adding 10 mL of sterilized miliQ water into the sealed chamber.

2.3. *DNA extraction, sequencing, and construction of reference genomes*

To obtain draft genomes for RNA read mapping, 5 mL *A. baumannii* or *K. pneumoniae* and 15 mL *Bacillus spp*. DNA was extracted using the DNeasy PowerSoil Pro Kit (Qiagen) following the manufacturer's instructions. DNA samples were submitted to the Microbial Genome Sequencing Center (MiGS) for library construction and sequencing. KneadData v0.7.10 (https://bitbucket.org/biobakery/kneaddata/) was then used for raw sequence quality control and contaminant removal with default parameters. Short reads were assembled into contigs using SPAdes v3.14.1[21] and each reference genome was annotated using Prokka v1.14.6[22]. Additional annotation of antibiotic and antimicrobial resistance genes were conducted using the Comprehensive Antibiotic Resistance Database (CARD)[23]. Assembled contigs from All Purpose Probiotic Cleaner were also binned into five metagenome-assembled genomes (MAGs) using metaWRAP[24]. CheckM[25] was used to assess the quality of microbial genomes. Taxonomic classification of each draft genome was determined by GTDB-Tk[26].

2.4. RNA Extraction, Sequencing, and Data Processing

Swab samples were stored in RNAprotect Bacteria Reagent under -80°C until extraction. RNA was extracted using the RNeasy Mini Kit (Qiagen) following its recommended "Protocol 2: Enzymatic lysis and mechanical disruption of bacteria", consisting of a 10 min treatment with 15 mg/mL lysozyme and 10 min beadbeating. Libraries were constructed at Northwestern NUSeq using the Illumina Total RNA Prep with Ribo-Zero Plus, according to manufacturers' protocol. A

158 total of 56 samples (54 samples collected at various time points and cleaning scenarios, one kit
159 control, and one sample prepared for future between-batch normalization). Samples were
160 sequenced using a HiSeq4000 with 50 bp single end reads.

161 Reference genomes constructed from whole genome sequencing were used for RNA mapping.
162 Raw sequence files were processed using trim_galore v0.6.6
163 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and Kneaddata v0.7.10
164 (https://bitbucket.org/biobakery/kneaddata/) to remove sequence adapters, low quality reads, and
165 contaminants. Sortmerna v4.2.0[27] was used to filter rRNA reads against the silva-bac-16s-id90
166 and silva-bac-23s-id98 databases. HISAT2 v2.1.0[28] was used to build local indices and map
167 short reads onto reference draft genomes. Transcript abundances were quantified using
168 featureCounts v2.0.1[29]. Differentially expressed genes (DEGs) were identified by DESeq2[30] at a
169 significance level of 0.05 (Benjamini-Hochberg adjusted p-value). BlastKOALA (KEGG
170 Orthology And Links Annotation) was used for K number assignment followed by Prokka
171 annotation[31]. KEGG pathway enrichment analysis was further conducted using ClusterProfiler[32].
172 Statistical analysis, including Welch's paired two sample *t*-test, principal component analysis
173 (PCA), and permutational multivariate analysis of variance (PERMANOVA, 9,999
174 permutations) were conducted in R (version 4.1.2, 2021-11-01).
175

176 ## 3. ***Results and Discussion***

177 ### ***3.1.*** *Survival of* A. baumannii *and* K. pneumoniae *under four cleaning scenarios and two*
178 *temperature/humidity conditions*

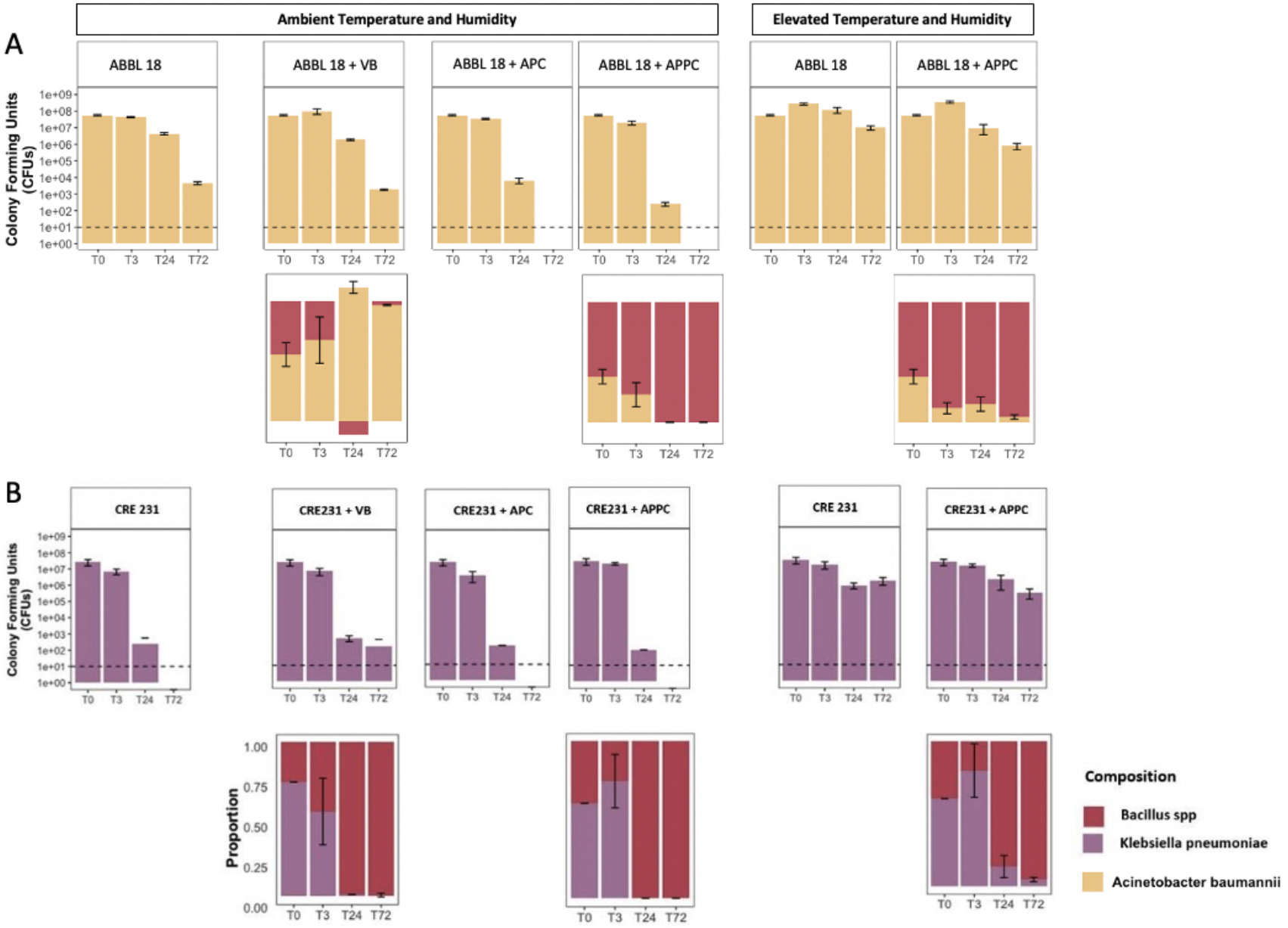
179 We quantified the colony forming units (CFUs) of *Acinetobacter baumannii* and *Klebsiella*
180 *pneumoniae* (using CHROM selective agar for *Acinetobacter baumannii* and MacConkey agar

for *Klebsiella pneumoniae*) to examine their survival under ambient and wet conditions in combination with four cleaning strategies: 1) no cleaning (ABBL18 or CRE231), 2) chemical surface cleaning with All-Purpose Cleaning (ABBL18 + APC or CRE231 + APC), 3) probiotic surface cleaning with All-Purpose Probiotic Cleaner (ABBL18 + APPC or CRE231 + APPC), 4) and probiotic surface cleaning with vegetative *Bacillus spp*. (ABBL18 + VB or CRE231 + VB). Under the circumstances where *Bacillus* were introduced through APPC and VB cleaning, total CFUs were quantified using TSA.

Over the course of 3 days, we observed a maximum $\log_{10}$ reduction of 8.75 for *A. baumannii* (ABBL18 + APC and ABBL18 + APPC under ambient condition) and 7.42 for *K. pneumoniae* (CRE231, CRE231 + APC and CRE231 +APPC). A minimum $\log_{10}$ reduction of 3.75 for *A. baumannii* (ABBL18 under elevated temperature and humidity) and 2.26 for *K. pneumoniae* (CRE231 under elevated temperature and humidity). Under ambient conditions, both APC and APPC demonstrated a near-complete inactivation of *A. baumannii* within 72 hours (<LOD) and complete inactivation for *K. pneumoniae*. Although the survival of *A. baumannii* between APPC and APC are not statistically significant ($p>0.05$, Figure S1), APPC cleaning at 24 hours showed 1.43 greater $\log_{10}$ reduction compared to APC (Figure 2A). The reduction of *A. baumannii* was largely attributed to the chemical component; however, APPC might be able to achieve a near-complete removal of *A. baumannii* within a shorter time frame compared to the use of chemical-based APC alone. Differences in the survival of *K. pneumoniae* between APPC, APC and no-cleaning scenarios was not statistically significant ($p>0.05$, Figure S1), with only 0.77 greater $\log_{10}$ reduction in APPC compared to APC at 24 hours. The fact that *K. pneumoniae* was unable to persist on the surface without any cleaning product under ambient conditions indicated that

chemical components in the cleaning product only had marginal contribution to *K. pneumoniae* inactivation.

Surfaces cleaned with APPC were also gradually dominated by *Bacillus spp*. due to the decreasing absolute abundance of the pathogen population. This persistence of *Bacillus spp*. on surfaces was observed under both ambient and elevated humidity and temperature (Figure 2). In contrast to the spores included in APPC, *Bacillus spp*. introduced as vegetative cells (ABBL18 + VB) were gradually taken over by the *A. baumannii* population. In contrast, for CRE231 + VB the cleaned surface was still dominated by *Bacillus spp* over *K. pneumoniae* (Figure 2A). Without the use of chemical-based surface cleaner (ABBL18 and ABBL18 + VB), *A. baumannii* showed comparable CFUs at all time points ($p > 0.05$). Samples with and without APPC cleaning under elevated temperature and humidity showed significantly greater viable populations of *A. baumannii* ($p<0.01$, Figure S1A), compared to ambient pairs. The same trend was observed for *K. pneumoniae* (Figure 2B), although paired *t*-tests showed low or no significance (Figure S1B). This result suggests greater potential health risks and infectious disease burdens in developing regions with a humid and hot climate and limited climate control[33].


A
Ambient Temperature and Humidity
Elevated Temperature and Humidity
ABBL 18
ABBL 18 + VB
ABBL 18 + APC
ABBL 18 + APPC
ABBL 18
ABBL 18 + APPC
Colony Forming Units (CFUs)
1e+09
1e+08
1e+07
1e+06
1e+05
1e+04
1e+03
1e+02
1e+01
1e+00
T0 T3 T24 T72
B
CRE 231
CRE231 + VB
CRE231 + APC
CRE231 + APPC
CRE 231
CRE231 + APPC
Proportion
1.00
0.75
0.50
0.25
0.00
Composition
Bacillus spp
Klebsiella pneumoniae
Acinetobacter baumannii

221

Figure 2. Colony forming units (CFU) of pathogens and composition of surface microbial community under four cleaning scenarios and two temperature and humidity conditions. CFUs of pathogens were counted on day 3 of incubation (37^{o}C) on their selective media while total CFUs (pathogen and *Bacillus spp.*) were obtained from TSA. The proportions of *A. baumannii* and *K. pneumoiae* were calculated as the ratio between CFU from their selective media and CFU from TSA. The proportion of *Bacillus spp.* was calculated as $\mathbf{1} - \frac{\boldsymbol{CFU(CHROM)}}{\boldsymbol{CFU(TSA)}}$. Dashed lines represent a limit of detection (LOD) of 10 CFU.

* Due to possible technical variation, the proportion of *A. baumannii* exceeded 1 at T24 for ABBL18 + VB.

229 *3.2. Overview of whole genome sequencing and metatranscriptomic data*

230 Sequencing of the *A. baumannii* (ABBL18) isolate generated 3.72 million reads with an average
231 read length of 136 bp. Reads were assembled into contigs with a N50 of 141,238 bp. Sequencing
232 of *K. pneumoniae* (CRE231) generated 4.4 million reads with an average rate of 145 bp. Reads
233 were assembled into contigs with a N50 of 113,698 bp. Sequencing of the All-Purpose Probiotic
234 Cleaner resulted in 16.49 million reads with an average read length of 139 bp, after quality
235 trimming and contaminant removal. Reads were assembled into contigs with a N50 of 143,313
236 bp. Open reading frame (ORF) identification recognized 4,039, 5,823, and 22,429 coding regions
237 for *A. baumannii, K. pneumoniae,* and APPC, respectively, 2,253, 4052, and 13,339 of which
238 were functionally annotated (not as "hypothetical protein") via Prokka. Among these annotated
239 regions for *A. baumannii, K. pneumoniae,* and APPC assemblies, 2,150, 3,675, and 11,594 were
240 classified into one or more KEGG Orthologs respectively. Contigs from APPC reads were
241 binned into five groups with completeness > 90%, and contamination < 5%. All five bins were
242 classified as *Bacillus* species as shown in Table 2.
243

244 Table 2. *Bacillus* genome binning and taxonomic assignment.

Bin	**Completeness (%)**	**Contamination (%)**	**GC (%)**	**Size (Mbp)**	**Taxonomic Classification**
1	90.58	1.883	0.351	4.74	*Bacillus cereus*
2	96.43	1.489	0.466	3.60	*Bacillus velezensis*
3	99.11	0.148	0.436	4.11	*Bacillus subtilis*

4	98.21	0.087	0.461	4.28	*Bacillus licheniformis*
5	99.08	0.207	0.416	3.57	*Bacillus safensis*

RNA samples containing *A. baumannii* or *K. pneumoniae* were collected for up to 72 hours of surface inoculation. Biological triplicates were collected for each condition and an average of 12.73 ± 3.83 and 11.03 ± 4.98 million reads with an average read length of 49 bp were recovered for *A. baumannii* and *K. pneumoniae* experiments, respectively, after removing low-quality reads, contaminated sequences, adapters, and rRNA. The average mapping rate using HISAT2 was 98.20% ± 1.06% for experiments with *A. baumannii* and 96.40% ± 10.24% for experiments with *K. pneumoniae* (Figure S2). Percentages of reads mapped onto *A. baumannii* and *Bacillus* genomes vary across four cleaning scenarios; VB samples containing vegetative *Bacillus spp.* on average had 40.53% ± 6.07% reads mapped onto *Bacillus* reference genomes, whereas this number for APPC samples was only 0.56% ± 0.75%. A similar trend was observed in experiments with *K. pneumoniae* and *Bacillus spp.* CRE231 + VB samples containing vegetative *Bacillus spp.* on average had 40.52% ±16.83% reads mapped onto *Bacillus* reference genomes; however, this number was only 1.00 ±1.08% for APPC samples. Thus, these data suggest that the majority of *Bacillus* in the probiotic product were likely to remain as spores throughout the inoculation. Due to this insufficient coverage of *Bacillus* transcriptome in APPC samples, our transcriptomic analysis is composed of quantitative comparisons for the *A. baumannii* and *K. pneumoniae* transcriptomes and qualitative evaluations for *Bacillus spp.*

3.3. *Transcriptomes of* A. baumannii *and* K. pneumoniae *clustered into distinct groups determined by cleaning and physical conditions*

Prior to conducting principal component analysis (PCA), read count data were transformed to remove the dependence of the variance on the mean. PCA of the transformed (variance stabilization) read counts belonging to *A. baumannii* indicated that the expression of *A. baumannii* clustered into three distinct groups based on cleaning scenarios and physical conditions (Figure 3A). Clustering of read counts from *K. pneumoniae* is more distinct for different time points and physical conditions (Figure S2) compared to cleaning scenarios and physical conditions (Figure 3C). PERMANOVA (9,999 permutations) revealed that temperature and humidity ($p<0.001$), cleaning ($p<0.001$), and time ($p<0.001$) explained 12%, 30%, and 9% of the variance for A. *baumannii* read counts, and 29%, 10%, and 12% for *K. pneumoniae* read counts, respectively. Gene expression profiles of *A. baumannii* with detergent cleaning (APPC and APC) are more similar to each other than they were to those without detergent (ABBL18 and VB). Temperature and humidity conditions also affected the expression of *A. baumannii* under the no-cleaning condition, but this impact was minimal when APPC was used. On the contrary, the temperature and humidity strongly affected the gene expression profiles of *K. pneumoniae* strongly, and the impact was more pronounced as the cleaning time extended from 3 hours to 24 and 72 hours.

Using no-cleaning samples (ABBL18 or CRE231) as the reference transcriptome, genes with absolute $\log_2$ fold change values greater than or equal to 1 as calculated by DESeq2 were considered as differentially expressed (Benjamini-Hochberg adjusted p-value ≤ 0.05). 414, 522,

and 634 genes of *A. baumannii* and 578, 145, and 57 genes of *K. pneumoniae* were found to be differentially expressed in APC, APPC, and VB samples, respectively. The transcriptome of *A. baumannii* with APC and APPC shared the highest level of similarity and consequently, the smallest number of differentially expressed genes (APPC vs APC, Figure 3B). In the case of *K. pneumoniae*, VB samples were the most similar to no-cleaning samples. Consistent with previous PCA analysis, expression of *A. baumannii* under ambient and elevated temperature and humidity conditions are drastically different from each other (1298 DEGs, Figure 3B) whereas this difference in transcriptome diminished when APPC was used (133 DEGs). The high impact of elevated temperature and humidity persisted for *K. pneumoniae* even when APPC was applied (1828 and 1838 DEGs with or without APPC, Figure 3D). Since the transcriptome of *K. pneumoniae* under APPC cleaning was fairly similar to the no-cleaning scenario, their similar response to elevated temperature and humidity could be expected. Samples collected at various time points throughout the microcosm inoculation also showed that much of the temporal variation in gene expression occurred within 24 hours of surface cleaning and the number of differentially expressed genes of the 72-hour samples decreased from 211 to 21 for A. *baumannii* and from 876 to 19 for *K. pneumoniae* when compared to 3-hour and 24-hour samples. Similar trends of differentially expressed gene numbers over time were observed in the read counts from vegetative *Bacillus* (Figure S4), where no gene was considered to be differentially expressed within 24 hours based on the criteria described above.

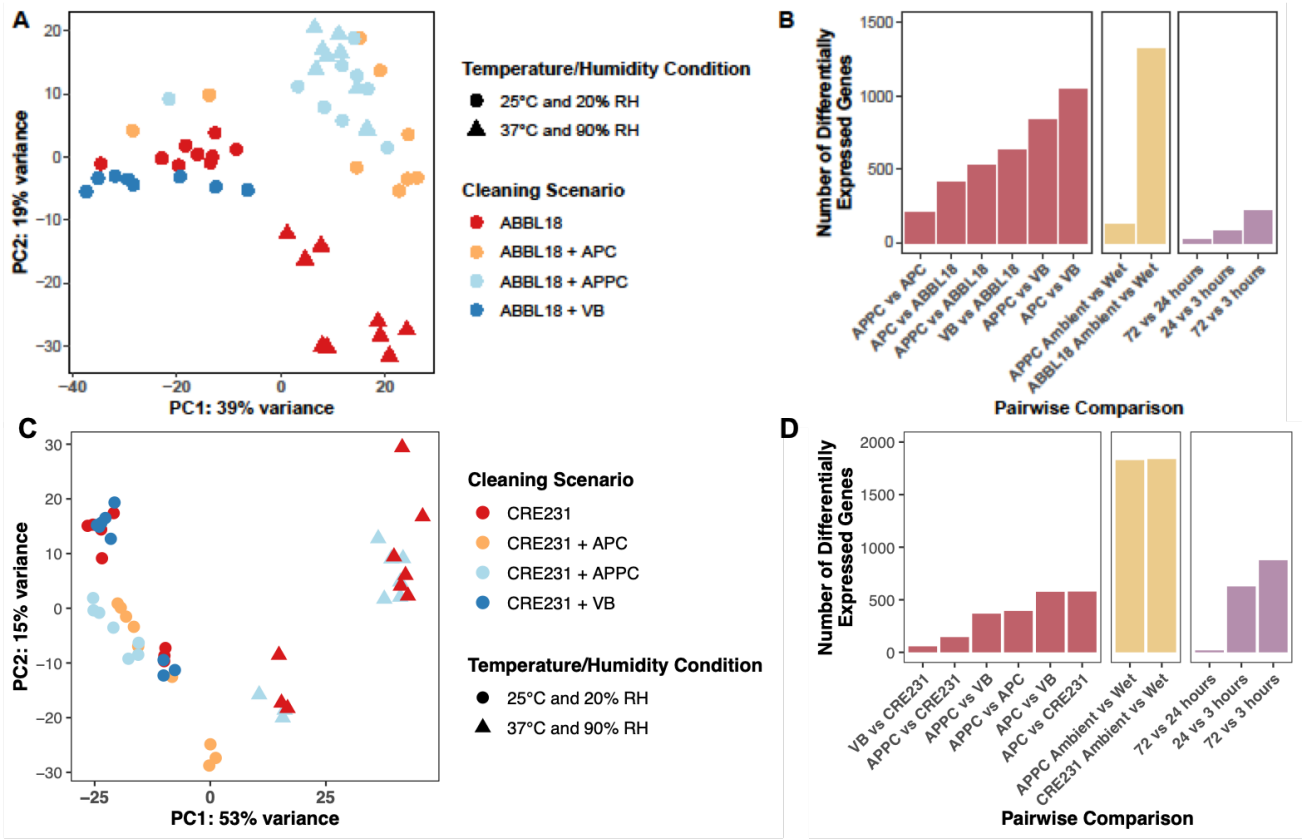


Figure 3. Principal component analysis on variance stabilized *Acinetobacter baumannii* (A) and *Klebsiella pneumoniae* (C) read counts and number of differentially expressed genes of 11 pairwise comparisons for *Acinetobacter baumannii* (B) and *Klebsiella pneumoniae* (D) experiments. Samples were color-coded based on cleaning conditions; each point (dot or triangle) represents its corresponding temperature and humidity condition.

311 *3.4. KEGG pathway enrichment profiles revealed different metabolic response under different*
312 *cleaning scenarios*
313
314 26 KEGG pathways related to metabolism (pyruvate, pyrimidine, purine, propionate, histidine,
315 fatty acids, etc.), translation (ribosome and aminoacyl-tRNA biosynthesis), energy production
316 (oxidative phosphorylation), and membrane transport (ABC transporters, and bacterial secretion
317 system) were positively enriched with a statistical significance in samples containing vegetative
318 *Bacillus* (ABBL18 + VB), compared to *A. baumannii* alone (ABBL18). Ribosome was the most
319 significant pathway, with 42 up-regulated genes. When vegetative *Bacillus spp.* were present,
320 protein synthesis of *A. baumannii* was likely promoted to achieve a large demand for surface
321 competitions. On the other hand, three pathways (geraniol degradation, fatty acid metabolism,
322 and valine, leucine, and isoleucine degradation) were positively enriched in samples with
323 chemical-based detergent cleaning (ABBL18 + APC and ABBL18 + APPC), indicating that the
324 *A. baumannii* population overall repressed metabolic activities and adaptively reduced synthesis
325 in response to environment constraints associated with detergent cleaning (Figure 4A).
326
327 11 KEGG pathways related to metabolism (citrate cycle, pyruvate, tyrosine, histidine, starch,
328 etc.), antibiotic resistance (cationic antimicrobial peptide resistance), and biofilm formation were
329 positively enriched with a statistical significance in samples containing vegetative *Bacillus*
330 (CRE231 + VB), compared to *K. pneumoniae* alone (CRE231). The TCA cycle was the most
331 significant pathway with 26 up-regulated genes (p-adjusted < 0.001, NES 2.36). Genes involved
332 in two-component systems and membrane transport (ABC) were upregulated in samples treated
333 with probiotic or detergent cleaners but not in samples containing vegetative *Bacillus* (CRE231).

This suggests that chemical detergents, rather than *Bacillus*, induce environmental sensing and response mechanisms in *K. pneumoniae*.

Differential expression of metabolic genes by *K. pneumoniae* was observed in each treatment group relative to no-cleaning controls. Carbon metabolism genes were up-regulated in each treatment group; however, the exact carbon processing pathways differed between groups. TCA cycle, pyruvate, starch and sucrose metabolism genes were up-regulated in samples containing vegetative *Bacillus* (CRE231 + VB) or detergent cleaner (CRE231 + APC) but not in samples treated with probiotic cleaner (CRE231 + APPC), suggesting a non-specific stress response. In contrast, no pathways were mutually and specifically enriched in samples containing vegetative *Bacillus* (CRE231 + VB) or probiotic cleaner (CRE231 + APPC), which is likely influenced by the diminished transcriptional landscape of *Bacillus* in probiotic cleaner (Figure S2). This suggests that vegetative *Bacillus* and cleaning products induce alternative though overlapping carbon metabolism networks in *K. pneumoniae*. Indeed, increased expression of biosynthetic pathways (glyoxylate cycle, carbon fixation) suggests vegetative *Bacillus* may promote anabolic competency in *K. pneumonia*. More research is needed to determine the role of metabolic manipulation with probiotics.

3.5. Different cleaning scenarios induced differential expression of genes associated with stress response and competition

Iron acquisition through siderophores is believed to be one of the key mechanisms of inter-species competitive exclusion[34]. Several genes responsible for siderophore synthesis, export, and

reception (e.g., aerobactin, anguibactin, and enterobactin) were slightly up-regulated in samples with detergent cleaning (Figure S5A) but down-regulated in samples containing vegetative *Bacillus spp*. in *A. baumannii* experiments. Although bacteria with multiple siderophore receptors can gain competitive advantages in social competition through siderophore cheating[35], the down-regulation of siderophore-related genes in these samples was likely due to utilization of iron originating from lysed cell debris, especially from *Bacillus spp*., rather than active competition through siderophore cheating, given the inability of vegetative *Bacillus spp*. to persist on surfaces (Figure 2A). For *K. pneumoniae* experiments, no siderophore-related gene was differentially expressed under the three cleaning scenarios (Figure S6A, *iutA*, *fepA*, and *fepC* $\log_2$ fold change absolute values < 1). The fact that *Bacillus* took over the surface in the presence of *K. pneumoniae* is likely related to *K. pneumoniae*'s inability to persist on the surface even without any cleaning (Figure 2B). Additionally, transformed *Bacillus spp*. read counts unveiled the predominant involvement of petrobactin as the primary siderophore in *Bacillus spp*. iron acquisition. Genes related to the synthesis, and export of *Acinetobacter*- and *Klebsiella*-associated siderophores (e.g., aerobactin, anguibactin, and enterobactin) were only occasionally expressed (Figure S7), further reducing the likelihood of having siderophore cheating on surfaces.

In addition to iron acquisition, genes related to Type VI Secretion Systems (T6SS), Type IV or Type 1 Pili, and biofilm formation were shown to have important implications in inter-species competition, virulence expression, and starvation response in *A. baumannii* or *K. pneumoniae* [34,36,37]. Genes associated with T6SS and Type IV Pili were slightly upregulated in APPC, APC, and VB samples for *A. baumannii* with varying statistical significance (Figure S5). Compared to

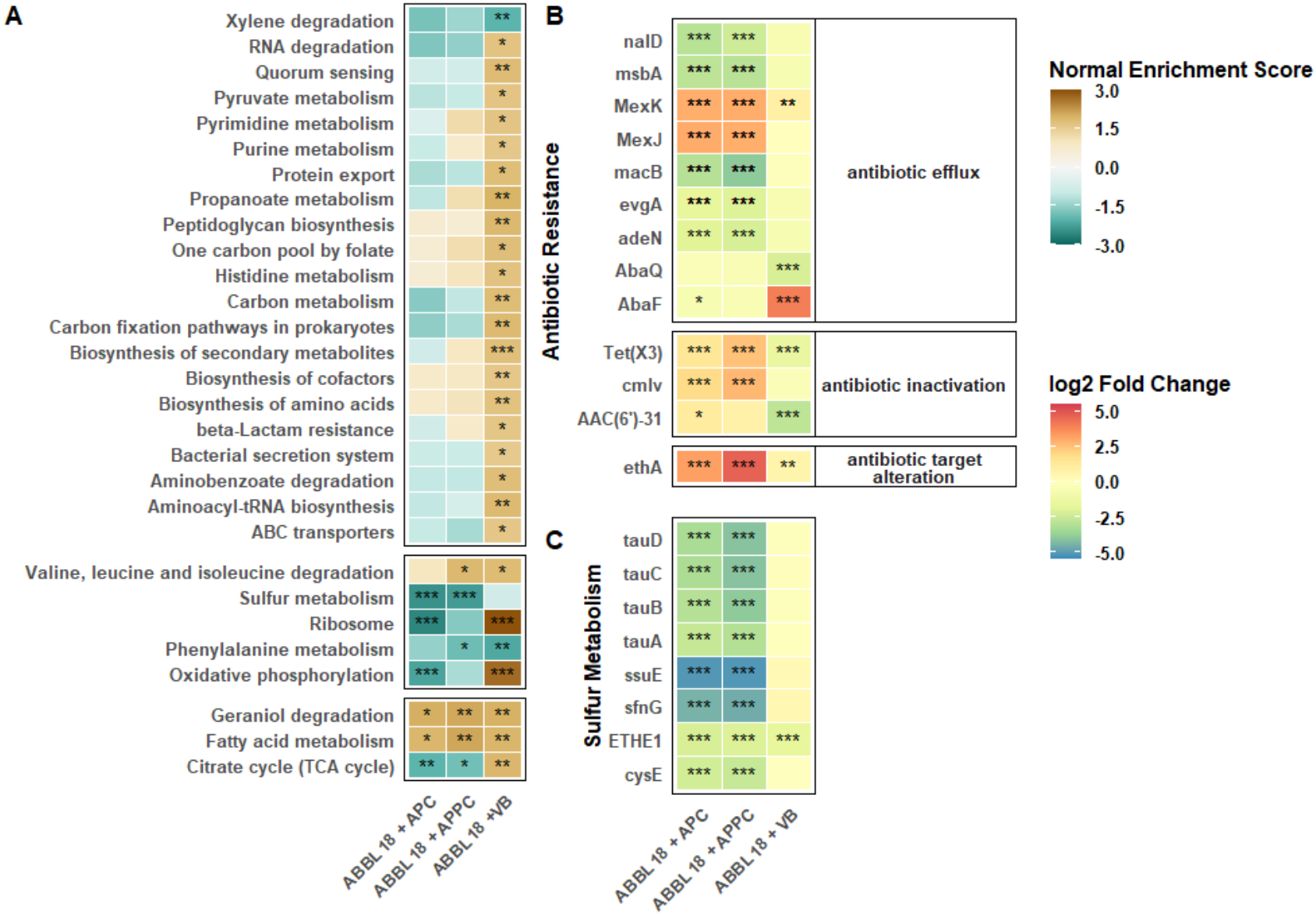
APC and APPC, a series of genes related to poly-beta-1,6-N-acetyl-D-glucosamine (PGA) production, a biofilm adhesin polysaccharide[38,39], were up-regulated in the presence of vegetative *Bacillus spp*. The increased expression of the *pgaABCD* operon with roles in surface binding and maintaining biofilm structure stability[38,39] suggested greater potential for PGA production and subsequent biofilm activities in samples containing both *A. baumannii* and *Bacillus spp*. In contrast, genes related to T6SS and Type 1 Pili had negligible differences under different cleaning scenarios for *K. pneumoniae* (Figure S6B,D). Additionally, only a few biofilm formation related genes were differentially expressed under different cleaning scenarios compared to non-cleaning samples for *K. pneumoniae*. Using absolute $\log_2$ fold change value ≥ 1 as threshold, eight genes were differentially expressed (four up-regulated, four down-regulated) in chemical cleaner (APC) samples, two genes were differentially expressed (one up-regulated, one down-regulated) in probiotic cleaning (APPC) samples, and five genes were differentially expressed (one up-regulated and four down-regulated) in vegetative *Bacillus* (VB) samples (Figure S7C). Lack of shared differentially expressed genes across cleaning scenarios with either chemical substances nor *Bacillus* indicated nonspecific response of *K. pneumoniae* biofilm formation towards different stressors. As biofilm formation is a complex process with an important role in pathogenesis, more detailed studies in light of biofilm formation prevention for various pathogenic species through different cleaning strategies are needed.

Meanwhile, several genes encoding efflux pumps were differentially expressed in APC and APPC samples with *A. baumannii*, including up-regulated *mexJ* and *mexK*, which encode the membrane fusion protein and the inner membrane resistance-nodulation-division (RND) transporter of the MexJK multidrug efflux protein[40] , as well as down-regulated transcriptional

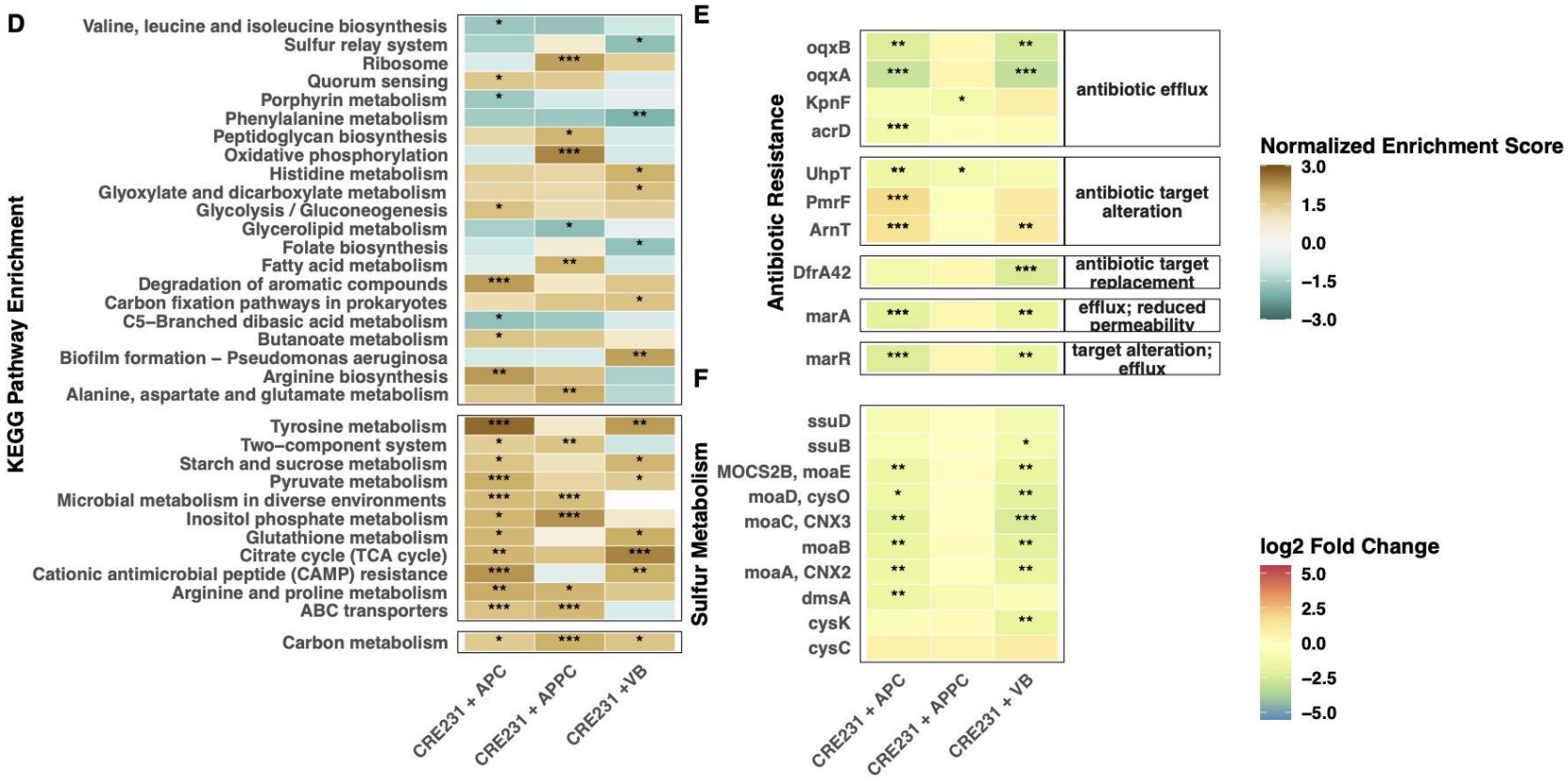
repressors of RND-type efflux pumps (e.g., *nalD*[41], and *adeN*[42]). In samples containing *A. baumannii* and vegetative *Bacillus spp*., we observed an increased expression of *abaF*, a gene encoding fosfomycin resistance. The effects of probiotic cleaner on *K. pneumoniae* antibiotic resistance gene expression were much less pronounced, as only the *kpnF* gene ($\log_2$ fold change = -1.09) belonging to major facilitator superfamily (MFS) antibiotic efflux pump and *uhpT* gene with mutation conferring resistance to fosfomycin were slightly down-regulated ($\log_2$ fold change = -1.01) in APPC samples (Figure 4E). However, no genetic determinants of fosfomycin biosynthesis were identified from *Bacillus spp*. reference genomes. Although bacterial multidrug resistance (MDR) efflux pumps are intensively studied for their profound impacts in reducing antibiotic and antimicrobial susceptibilities, efflux pumps also alter transcriptomic response related to central metabolism, pathogenicity, environmental sensing, and stress response[43]. For example, the disruption of *abaF* in *A. baumannii* not only resulted in an increase in fosfomycin susceptibility but also a decrease in biofilm formation and virulence[44]. *arnT* and *pmrF* genes associated with peptide antibiotic resistance were up-regulated in APC samples for *K. pneumoniae*, which could be induced by the chemical cleaning product.

Lastly, both APC and APPC showed pronounced downregulation of genes related to sulfur metabolism, particularly those induced by sulfur starvation, but the expression of these genes remained constant when no detergent was used for *A. baumannii* (Figure 4C). These genes include a NADPH-dependent FMN reductase (*ssuE*) that was down-regulated by 35.13 fold in APPC and 34.62 fold in APC, and upregulated by 1.30 fold in VB (adjusted p-value > 0.05), as well as a *tauABCD* operon known to facilitate the utilization of taurine as an alternative sulfur source under sulfate-deprived environment[45]. Similar down-regulation of sulfonate assimilation

426 genes (*ssuA*, *ssuB*, *ssuC*, *ssuD*, etc.) was observed under elevated temperature and humidity
427 conditions with APPC (Figure 5C). Owing to the presence of anionic surfactants in liquid
428 detergent products, such as sodium lauryl ether sulfate (SLES)[46], these sulfate starvation genes
429 were likely suppressed in APC and APPC samples. This observation is consistent with studies
430 that illustrated the overexpression of *tauABCD* and *ssuEADCB* genes induced by sulfate
431 shortage[47]. Additionally, bacterial utilization of SLES facilitated by *Citrobacter braakii* [48] and a
432 consortium of *Acinetobacter calcoacetiacus*, *Klebsiella oxytoca*, and *Serratia odorifera*[46] has
433 also been reported in previous studies and may contribute to the persistence of microbial
434 communities associated with hospital sinks[49]. The molybdenum cofactor biosynthesis operon
435 genes *moaABCDE*[50] were slightly down-regulated in APC and VB samples but not in APPC
436 samples for *K. pneumoniae* (Figure 4F). Most sulfur metabolism and antimicrobial resistance
437 related genes were not differentially expressed in APPC samples for *K. pneumoniae* but were
438 differentially expressed at different levels of significance in APC or VB samples. It is possible
439 that the dormant *Bacillus* cells in APPC were buffering the stress caused by chemical cleaner
440 while the vegetative *Bacillus* cells introduced competition pressure on the surfaces, as mentioned
441 previously. Further studies on the inter-species interaction between dormant or vegetative
442 *Bacillus* with different pathogens are needed to facilitate the development of probiotic cleaning
443 strategies.


A
Xylene degradation
RNA degradation
Quorum sensing
Pyruvate metabolism
Pyrimidine metabolism
Purine metabolism
Protein export
Propanoate metabolism
Peptidoglycan biosynthesis
One carbon pool by folate
Histidine metabolism
Carbon metabolism
Carbon fixation pathways in prokaryotes
Biosynthesis of secondary metabolites
Biosynthesis of cofactors
Biosynthesis of amino acids
beta-Lactam resistance
Bacterial secretion system
Aminobenzoate degradation
Aminoacyl-tRNA biosynthesis
ABC transporters
Valine, leucine and isoleucine degradation
Sulfur metabolism
Ribosome
Phenylalanine metabolism
Oxidative phosphorylation
Geraniol degradation
Fatty acid metabolism
Citrate cycle (TCA cycle)
ABBL 18 + APC
ABBL 18 + APPC
ABBL 18 +VB
B
Antibiotic Resistance
naID
msbA
MexK
MexJ
macB
evgA
adeN
AbaQ
AbaF
antibiotic efflux
Tet(X3)
cmlv
AAC(6')-31
antibiotic inactivation
ethA
antibiotic target alteration
C
Sulfur Metabolism
tauD
tauC
tauB
tauA
ssuE
sfnG
ETHE1
cysE
ABBL 18 + APC
ABBL 18 + APPC
ABBL 18 + VB
Normal Enrichment Score
3.0
1.5
0.0
-1.5
-3.0
log2 Fold Change
5.0
2.5
0.0
-2.5
-5.0

444


D
KEGG Pathway Enrichment
Valine, leucine and isoleucine biosynthesis
Sulfur relay system
Ribosome
Quorum sensing
Porphyrin metabolism
Phenylalanine metabolism
Peptidoglycan biosynthesis
Oxidative phosphorylation
Histidine metabolism
Glyoxylate and dicarboxylate metabolism
Glycolysis / Gluconeogenesis
Glycerolipid metabolism
Folate biosynthesis
Fatty acid metabolism
Degradation of aromatic compounds
Carbon fixation pathways in prokaryotes
C5–Branched dibasic acid metabolism
Butanoate metabolism
Biofilm formation – Pseudomonas aeruginosa
Arginine biosynthesis
Alanine, aspartate and glutamate metabolism
Tyrosine metabolism
Two–component system
Starch and sucrose metabolism
Pyruvate metabolism
Microbial metabolism in diverse environments
Inositol phosphate metabolism
Glutathione metabolism
Citrate cycle (TCA cycle)
Cationic antimicrobial peptide (CAMP) resistance
Arginine and proline metabolism
ABC transporters
Carbon metabolism
CRE231 + APC
CRE231 + APPC
CRE231 +VB
E
Antibiotic Resistance
oqxB
oqxA
KpnF
acrD
antibiotic efflux
UhpT
PmrF
ArnT
antibiotic target alteration
DfrA42
antibiotic target replacement
marA
efflux; reduced permeability
marR
target alteration; efflux
F
Sulfur Metabolism
ssuD
ssuB
MOCS2B, moaE
moaD, cysO
moaC, CNX3
moaB
moaA, CNX2
dmsA
cysK
cysC
CRE231 + APC
CRE231 + APPC
CRE231 + VB
Normalized Enrichment Score
3.0
1.5
0.0
−1.5
−3.0
log2 Fold Change
5.0
2.5
0.0
−2.5
−5.0

445

Figure 4. KEGG pathway enrichment results for samples collected under ambient temperature and humidity condition (A, D), log_2 fold change of genes associated with antibiotic/antimicrobial resistance (B, E), and sulfur metabolism (C, F), compared to no-cleaning samples (ABBL18 or CRE231). KEGG pathways shown here were statistically enriched in at least one of the cleaning conditions (Benjamini-Hochberg corrected p-value ≤ 0.05). Normalized enrichment score (NES) accounts for differences in gene set size and were used to compare enrichment results across KEGG pathways; a positive NES suggests an overall positive up-regulation of genes belonging to its corresponding pathway[32,51,52]. * p<0.05, ** p< 0.01, and ***p < 0.001.

3.6. *Effects of elevated temperature and humidity on pathogen cell motility and biofilm formation*

Although over 1,000 differentially expressed genes were identified from *A. baumannii* or *K. pneumoniae* transcriptomes between the two temperature and humidity conditions, the majority of the KEGG pathways were composed of genes that were both up-regulated and down-regulated, rendering non-significant results for most pathways. Several exceptions include xylene degradation and oxidative phosphorylation, which were down-regulated in *A. baumannii* samples (Figure 5A, ABBL18 Wet vs ABBL18 Ambient); pentose and glucuronate interconversions, phenylalanine metabolism, quorum sensing, which were up-regulated; and histidine, inositol phosphate, and porphyrin metabolism, which were down-regulated in *K. pneumoniae* samples (Figure 5D, CRE231 Wet vs CRE231 Ambient).

Under the elevated temperature and humidity condition, the *A. baumannii* transcriptome with APPC was positively enriched with four pathways related to energy production (oxidative phosphorylation), biosynthesis of secondary metabolites (e.g., aerobactin) and metabolism (e.g., propanoate, glyoxylate, and dicarboxylate). Metabolic pathways, such as toluene, benzoate, chlorocyclohexane, and chlorobenzene, were suppressed (Figure 5A, APPC Wet vs ABBL18 Wet). Under elevated temperature and humidity, 16 KEGG pathways related to metabolism (10), membrane transport (3), biosynthesis (2) and translation (1) were enriched in samples with probiotic cleaner (APPC) compared to no-cleaning *K. pneumoniae* samples. Pathways related to valine, leucine and isoleucine biosynthesis and sulfur metabolism were suppressed (Figure 5D, APPC Wet vs CRE231 Wet). This corresponded to the PCA and PERMANOVA results where environmental conditions (temperature and humidity) affected the transcriptome profiles of *K. pneumoniae* more than *A. baumannii*.

In addition to the impact on sulfur metabolism (Figure 5C and Figure 4C) discussed in the previous section, increased moisture availability likely resulted in an increase in *A. baumannii* cell motility through Type IV Pili (Figure 5B) without APPC. Increased moisture availability might also induce a downregulation of osmo-regulation genes such as *envZ* (osmolarity sensor protein, $\log_2$ fold change = -1.38) and *betT2* (osmo-dependent choline transporter $\log_2$ fold change = -2.14). Type 1 pili help mediate colonization by pathogens and promotes host-pathogen interaction as an important virulence factor[53,54]. Some type 1 pilus genes (fimA_1, fim, and smf-1) were expressed in *K. pneumoniae*, but none were found to be differentially expressed under different cleaning scenarios or under elevated temperature and humidity (Figure S6D). Although elevated temperature and humidity did not affect pilus-associated genes, 19 genes related to

biofilm formation were differentially expressed in *K. pneumoniae* samples (Figure 5D, CRE231 Wet vs CRE231 Ambient), with 14 down-regulated and 5 up-regulated genes. In addition, the quorum sensing pathway, which affects biofilm formation of *K. pneumoniae*[55] , was enriched under elevated temperature and humidity (Figure 5E, CRE231 Wet vs CRE231 Ambient). This indicated that elevated temperature and humidity could assist the persistence of *K. pneumoniae* on surfaces (Figure 2B) by altering the biofilm formation related gene expression. The effects of APPC on *K. pneumoniae* were likely to be masked by the changes due to higher temperature and humidity (Figure 5E), meaning the benefits of probiotic cleaning products could be more limited in tropical areas.

Our results demonstrated the persistence of probiotic *Bacillus* included in the All-Purpose Probiotic Cleaner up to 72 hours after cleaning, owing to the ability of *Bacillus* spores to survive unfavorable conditions. *A. baumannii* on surfaces cleaned using chemical-based detergent with and without probiotic *Bacillus* contained a comparable amount of viable pathogens, possibly due to the small percentage of germinated *Bacillus* cells and/or slow germination rate on surfaces. The transcriptome of *A. baumannii* with and without probiotic addition shared a high degree of similarity in overall gene expression, including reduced material synthesis and surface activities. On the contrary, the transcriptome of *K. pneumoniae* with probiotic addition showed a high degree of differences compared to samples with chemical cleaner or vegetative *Bacillus*, including reduced response in genes related to antimicrobial resistance, sulfur metabolism and biofilm formation. Longer persistence of viable pathogens on surfaces and reduced effects of APPC on *K. pneumoniae* overall gene expressions with and without cleaners under elevated temperature and humidity suggests that vigilant indoor climate control could contribute to

514 infection prevention in healthcare-associated scenarios. As the idea of using probiotic
515 microorganisms to promote health benefits becomes increasingly attractive in the field of
516 household cleaning, current understanding on the mechanism and implication of probiotic
517 cleaning remains limited.
518
519 ***Acknowledgement***
520
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522 004298. Under the grant conditions of the Foundation, a Creative Commons Attribution 4.0
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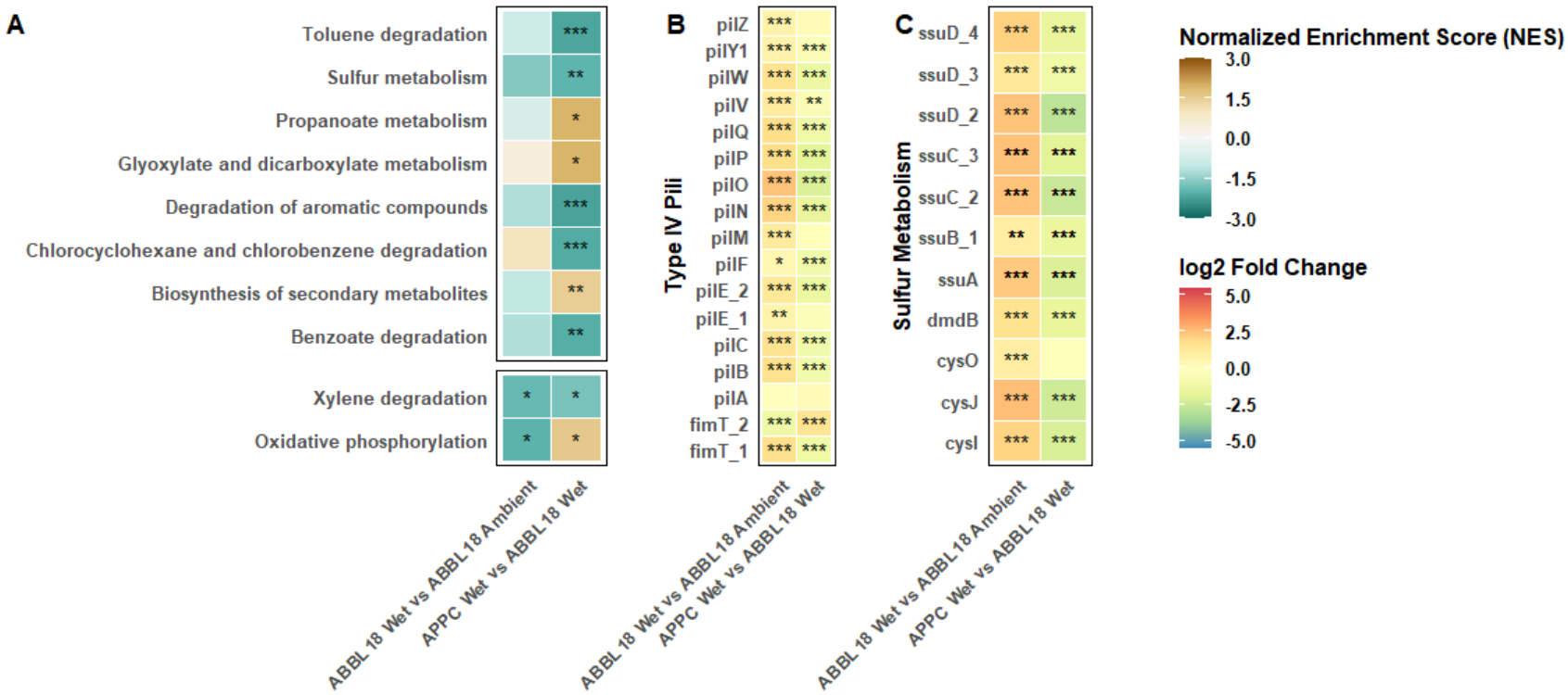
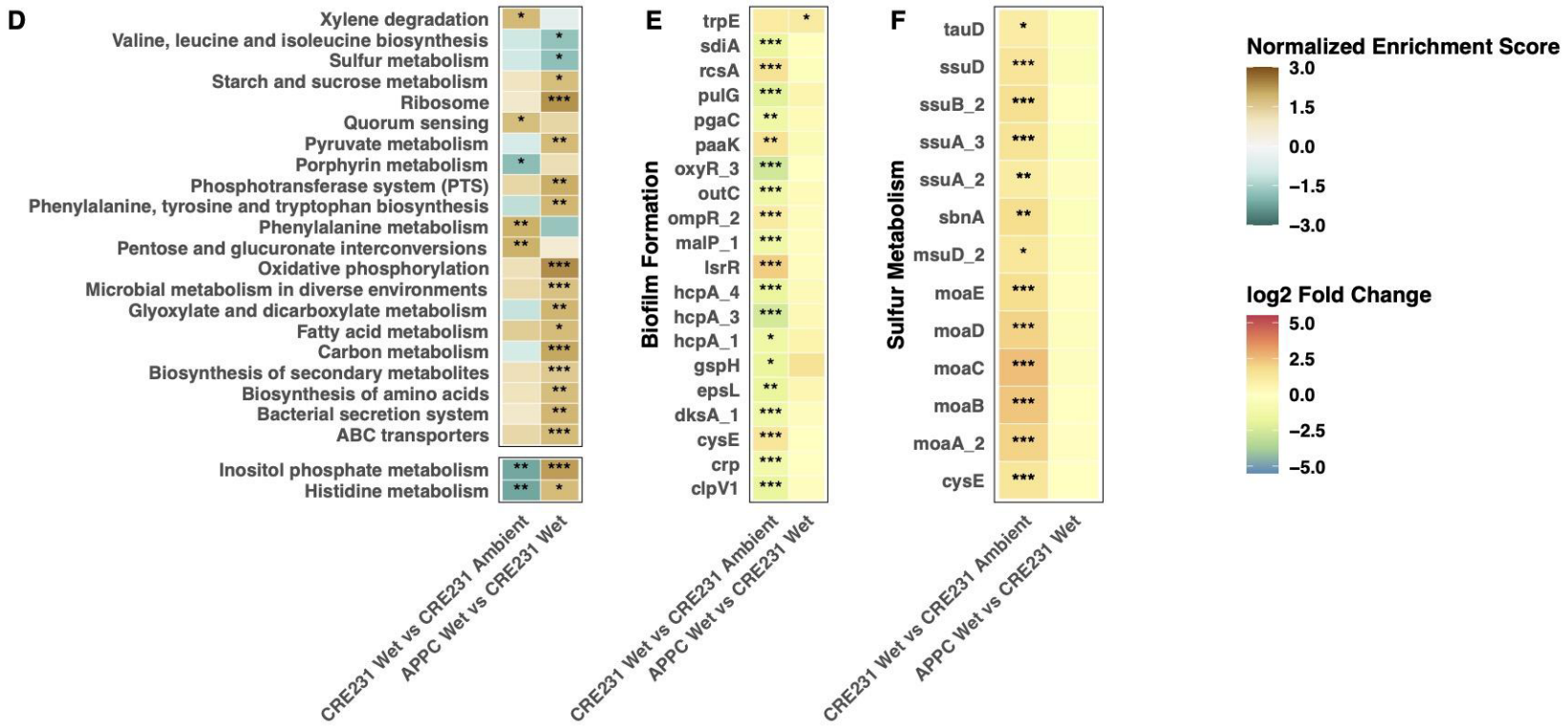
526 Marina Zelivyanskaya and Dr. Alan R. Hauser for providing clinical isolates of *Acinetobacter*
527 *baumannii* and *Klebsiella pneumoniae*. We further thank Andreas Wagner for his assistance.


A
Toluene degradation
Sulfur metabolism
Propanoate metabolism
Glyoxylate and dicarboxylate metabolism
Degradation of aromatic compounds
Chlorocyclohexane and chlorobenzene degradation
Biosynthesis of secondary metabolites
Benzoate degradation
Xylene degradation
Oxidative phosphorylation
ABBL 18 Wet vs ABBL 18 Ambient
APPC Wet vs ABBL 18 Wet
B
Type IV Pili
pilZ
pilY1
pilW
pilV
pilQ
pilP
pilO
pilN
pilM
pilF
pilE_2
pilE_1
pilC
pilB
pilA
fimT_2
fimT_1
C
Sulfur Metabolism
ssuD_4
ssuD_3
ssuD_2
ssuC_3
ssuC_2
ssuB_1
ssuA
dmdB
cysO
cysJ
cysI
Normalized Enrichment Score (NES)
3.0
1.5
0.0
-1.5
-3.0
log2 Fold Change
5.0
2.5
0.0
-2.5
-5.0

528



529

530 **Figure 5.** KEGG pathway enrichment results for no-cleaning and APPC samples collected under elevated temperature

531 and humidity condition (A, D), $\log_2$ fold change of genes associated with *Acinitobacter baumannii* cell motility (B),

532 *Klebsiella pneumonia*e biofilm formation (E), and sulfur metabolism (C, F). KEGG pathways shown here were statistically

533 enriched in at least one of the cleaning conditions (Benjamini-Hochberg corrected p-value ≤ 0.05). * p<0.05, ** p< 0.01,

534 ***p < 0.001.

535

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537

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