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

Indoor surfaces are paradoxically presumed to be both colonized by pathogens, necessitating disinfection, and "microbial wastelands." In these resource-poor, dry environments, competition and decay are thought to be important drivers of microbial community composition. However, the relative contributions of these two processes have not been specifically evaluated. To bridge this knowledge gap, we used microcosms to specifically evaluate whether interspecies interactions occur on surfaces. We combined transcriptomics and traditional microbiology techniques to investigate whether competition occurred between two clinically important pathogens, *Acinetobacter baumannii* and *Klebsiella pneumoniae*, and a probiotic cleaner containing a consortium of *Bacillus* species. Probiotic cleaning seeks to take advantage of ecological principles such as competitive exclusion, thus using benign microorganisms to inhibit viable pathogens, but there is limited evidence that competitive exclusion in fact occurs in environments of interest (i.e., indoor surfaces). Our results indicate that competition in this setting has a negligible impact on community composition but may influence the functions

expressed by active organisms. Although *Bacillus* spp. remained viable on surfaces for an extended period of time after application, viable colony forming units (CFUs) of *A. baumannii* recovered following exposure to a chemical-based detergent with and without *Bacillus* spp. showed no statistical difference. Similarly, for *K. pneumoniae*, there were small statistical differences in CFUs between cleaning scenarios with or without *Bacillus* spp. in the chemical-based detergent. The transcriptome of *A. baumannii* with and without *Bacillus* spp. exposure shared a high degree of similarity in overall gene expression, but the transcriptome of *K. pneumoniae* differed in overall gene expression, including reduced response in genes related to antimicrobial resistance. Together, these results highlight the need to fully understand the underlying biological and ecological mechanisms for community assembly and function on indoor surfaces, as well as having practical implications for cleaning and disinfection strategies for infection prevention.

Keywords: inter-species interactions; *Bacillus*; *Acinetobacter baumannii*; *Klebsiella pneumoniae*; transcriptomic response; indoor surface cleaning; infection prevention

38 ## *1. Introduction*

39

40 Indoor surfaces harbor pathogens and mediate the transmission of infectious disease. At the same
41 time, these environments have been called a "microbial wasteland" where microbes are "dead,
42 dying, or dormant." Following this model, surface-associated microbial communities are almost
43 entirely driven by the opposing forces of dispersal and death. Surface cleaning and disinfection
44 practices seek to promote microbial cell death, thereby preventing infection, especially in
45 healthcare settings. While cleaning using antimicrobials can effectively reduce microbial load on
46 contaminated surfaces in health care facilities[1], evidence of their effects on preventing disease
47 transmission and reducing healthcare-acquired infections remain limited[2]. Moreover, because
48 antimicrobials can cause both acute and chronic health impacts[3] and lead to antimicrobial
49 resistance[4–6], there is a strong drive to develop alternative cleaning strategies.

50

51 Probiotic cleaning seeks to take advantage of competitive exclusion and other ecological
52 principles to inhibit viable pathogens on surfaces[4,7]. Members of *Bacillus*, *Bifidobacterium*,
53 *Lactobacillus*, *Rhodopseudomonas*, and *Saccharomyces* are the most frequently used
54 microorganisms in these microbial based cleaning products[8]. While probiotic organisms may
55 persist on surfaces, competitive exclusion is not compatible with the "wasteland" model, which
56 stipulates no growth or metabolic activity. Previous studies of probiotic cleaners have shown that
57 probiotic cleaning decreases the recovery of cultivable bacterial pathogens[97], and even
58 inactivates viruses[10]. Competitive exclusion is an unlikely mechanism of action against viruses,
59 but even for bacteria there is limited direct evidence documenting competitive exclusion[11].
60 Rather, culture-based results support the hypothesis that probiotic *Bacillus* outcompete

61 pathogens in some environmental conditions but are in fact out-competed by resident organisms
62 when the latter are present in biofilms[11]. Moreover, there is a dearth of molecular evidence
63 supporting whether surviving probiotic organisms and healthcare-associated pathogens are
64 engaged in activities such as nutrient scavenging, stress response, or microbe-microbe
65 interactions[11]. To this end, studying gene expression levels instead of cultivable colonies or gene
66 presence in both pathogens and probiotic microorganisms is necessary to unravel the
67 mechanisms of probiotic cleaning. Transcriptomics allows quantitative determination of changes
68 in regulation of genes and pathways of the microorganisms upon application of probiotic
69 cleaners under different conditions, revealing changes in function and metabolic activity. Despite
70 these advantages, the application of transcriptomics in indoor surface microbiome studies has
71 been sparse[12]. Current indoor surface microbiome studies only focus on taxonomic profiling and
72 functional potentials that recovered from (meta)genomics analysis where the importance of gene
73 expression levels is overlooked or yet to be addressed[13,14]. Transcriptomics analysis from this
74 study can provide insights on the response of pathogens to tested cleaning scenarios on indoor
75 surfaces.
76
77 *Acinetobacter baumannii* and *Klebsiella pneumoniae* have been recognized as a major source of
78 healthcare-associated infections associated with high morbidity and mortality rates due to their
79 resistance to many antibiotics including carbapenems[15,16]. These organisms not only persist on
80 surfaces for up to several months[17] but are commonly found in healthcare environments,
81 especially in low- and middle-income countries. *K. pneumoniae* have been found on surfaces in
82 hospitals in Brazil[16,15], Tunisia[17], Russia[18], China[19] and Ghana[20] *A. baumannii* was found on
83 surfaces in hospitals in Brazil[21], Turkey[21], Saudi Arabia[22] and on the surfaces of medical devices

84 in Iran[23]. It was also found on surfaces in buildings surrounding hospitals in the US[24]. Both *A.*
85 *baumannii* and *K. pneumoniae* were found on surfaces in neonatal intensive care units in Egypt[25]
86 and hospitals in Algeria[26]. While these organisms likely reach surfaces via dispersion from
87 human occupants, they can also be transferred back to humans, particularly the very vulnerable.
88 *K. pneumoniae* persists in neonatal intensive care units and transfers to neonates' gut[27,28] and
89 possibly also the nares[29]. A high degree of relatedness has been observed between genotypes of
90 *A. baumannii* from patients and their surrounding rooms[30], and bidirectional transfer of *A.*
91 *baumannii* between the environment and adult patients has also been observed[31]. Notably in this
92 last study, *A. baumannii* then persisted in the environment despite terminal disinfection[31]. More
93 concerning than individual transfer events or colonization, surface-associated organisms can
94 cause or exacerbate outbreaks within a hospital. For example, surface-associated *K. pneumoniae*
95 was associated with an outbreak in Vietnam[32], and *A. baumannii* on bed railings was associated
96 with an outbreak in Saudi Arabia[33].
97
98 To understand how these organisms survive on surfaces, sometimes in defiance of cleaning
99 efforts, we sought to observe changes in gene expression in *A. baumannii* and *K. pneumoniae* on
100 surfaces with and without cleaning. Sequencing of low biomass samples collected from the built
101 environment often suffers from a low signal-to-noise ratio[34]. To obtain a fundamental
102 mechanistic understanding of indoor cleaning strategies and their impacts on microbial survival
103 and interactions, we utilized microcosm chambers containing stainless steel sheets to simulate a
104 simplified built environment with greater than usual microbial load. Because antimicrobial
105 resistance and hospital-acquired infections disproportionately affect low- and middle-income
106 countries, many of which are located in tropical climates, we also evaluated the effects of

107 elevated temperature and relative humidity (RH). To evaluate general ecological phenomena,
108 and particularly competitive exclusion, governing survival in mixed communities, we further
109 exposed these organisms to a probiotic cleaner containing several strains of *Bacillus*.
110

111 ## 2. Materials and Method

112 ### *2.1. Microcosm set-up*

113

114 A commercially available All-Purpose Probiotic Cleaner (referred to as probiotic cleaner herein)
115 was obtained from Graz, Austria. Clinical isolates of *A. baumannii* (ABBL18) and *K.*
116 *pneumoniae* (CRE231) were provided by Dr. Alan Hauser, Northwestern University Feinberg
117 School of Medicine. To simulate a typical high-touch surface in a healthcare environment, 24G
118 stainless steel sheets were cut into 2"x2" squares. *A. baumannii* and *K. pneumonia*e isolates were
119 grown in 40 mL tryptic soy broth (TSB) for 23 hours at 30°C with continuous shaking to its mid
120 log phase. The probiotic cleaner was filtered through a 0.22 μm syringe filter to create an
121 Cleaner Only control without probiotic materials. Sterility of the Cleaner Only control was
122 assessed by plating on tryptic soy agar (TSA). *Bacillus* spp. were germinated by mixing 0.1%
123 probiotic cleaner with TSB for use as a model Germinated *Bacillus* control where cells were
124 vegetative. This culture was incubated at 25°C for 23 hours with continuous shaking. Cells
125 (*Bacillus* spp., *A. baumannii* and *K. pneumoniae*) were harvested and washed in phosphate-
126 buffered saline (PBS) before inoculation. A total of six testing scenarios for each pathogen
127 (twelve groups in total) were specified as shown in Table 1:
128

129 **Table 1.** Testing scenarios and their experimental group names for each pathogen (i.e.,
130 *A. baumannii* and *K. pneumoniae)* consisting of four cleaning conditions, and two
131 temperature and humidity conditions. Cleaning conditions include probiotic-containing
132 and probiotic-free All-Purpose Probiotic Cleaner solutions, vegetative *Bacillus* spp. as a
133 model probiotic, and a no cleaning (i.e., pathogen alone) control. Ambient and elevated
134 temperature and relative humidity (referred to as Ambient and Humid respectively)
135 conditions were assessed as environmental variables. N/A means the scenario was not
136 tested.

Cleaning Scenarios	**Temperature and humidity conditions**	
	25°C and 20% RH	**37°C and 90% RH**
Pathogen only	No Cleaner (Ambient)	No Cleaner (Humid)
Pathogen + filtered probiotic cleaner	Cleaner Only	N/A
Pathogen + probiotic cleaner	Probiotic Cleaner (Ambient)	Probiotic Cleaner (Humid)
Pathogen + germinated *Bacillus*	Germinated *Bacillus*	N/A

137

138

139 Each 2”x2” stainless steel surface was first inoculated with 50 μL *A. baumannii* (corresponding
140 to 8.5×10^7 CFU) or 50 μL *K. pneumoniae* (corresponding to 1.0×10^8 CFU). 50 μL sterilized PBS
141 buffer, filtered probiotic cleaner, probiotic cleaner, or vegetative *Bacillus* spp. inoculum was

142 added to create No Cleaner control samples (*A. baumannii* or *K. pneumoniae* only), Cleaner
143 Only groups (chemical cleaning samples), Probiotic Cleaner groups (probiotic cleaning samples),
144 or Germinated *Bacillus* groups (vegetative cleaning samples), respectively. Following the
145 introduction of bacterial inoculum, all surfaces were transferred into autoclaved mason jars
146 (referred to as microcosm chambers) under two treatment conditions: 1) ambient temperature
147 (25°C) and relative humidity (20%), and 2) elevated temperature (37°C) and relative humidity
148 (>90%) with 10 mL of autoclaved milliQ water as in[13]. The relative humidity was measured
149 using Fisherbrand™ Traceable™ Humidity-On-A-Card™ Humidity Monitor (Waltham, MA,
150 USA). Each microcosm chamber contained one surface sample. All microcosm chambers were
151 stored in incubators maintained at 25°C or 37°C. Samples were sacrificed and biomass recovered
152 by swabbing at three specific timepoints: 3 hours, 24 hours, and 72 hours following the
153 inoculation and cleaning. Triplicate samples were prepared for each combination of cleaning,
154 timepoint, and incubation conditions (Figure 1).

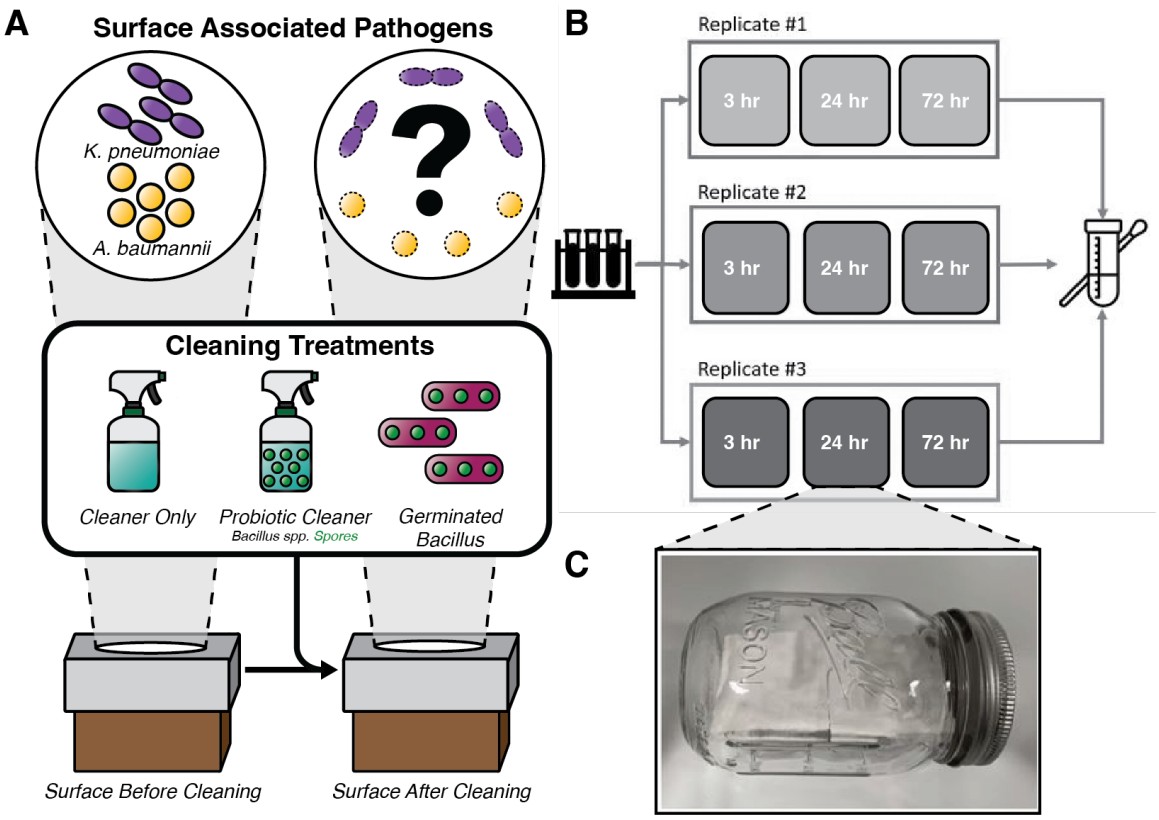


Figure 1. General overview of the biological question (A). Microcosm experimental design (B) with four types of inocula containing pathogen (*A. baumannii* or *K. pneumoniae*) and *Bacillus* spp. and sealed sterile microcosm chamber maintained under ambient temperature and relative humidity (C). The elevated temperature and humidity condition was maintained by adding 10 mL of sterilized miliQ water into the sealed chamber.

2.2. Swab sample collection

Swab samples were collected through a combination of dry and wet swabbing as performed previously[13]. 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 colony

168 forming units (CFUs) enumeration and RNA extraction, respectively. Surfaces were then wet
169 swabbed for a total of 20 s, including 5 seconds of rinse and rewet. Finally, the swabs were left
170 in 15 mL conical tubes and vortexed for 10 seconds. For total enumeration, 100 μL swab
171 suspension along with its dilutions were spread onto TSA. Additionally, the same volume of
172 swab suspension and dilutions were spread onto CHROM *A. baumannii* selective agar or on
173 MacConkey agar (as *K. pneumoniae* selective media[35]). Under the circumstances where *Bacillus*
174 were introduced through Probiotic Cleaner and Germinated *Bacillus* cleaning, total CFUs were
175 quantified using TSA. All plates were incubated at 37°C. Numbers of CFUs were counted after
176 three days.
177
178 2.3. *DNA extraction, sequencing, and construction of reference genomes*
179
180 To obtain draft genomes for RNA read mapping, 5 mL *A. baumannii* or *K. pneumoniae* and 15
181 mL *Bacillus* spp. DNA was extracted using the DNeasy PowerSoil Pro Kit (Qiagen) following
182 the manufacturer's instructions. DNA samples were submitted to the Microbial Genome
183 Sequencing Center (MiGS) for library construction and short-read sequencing on an Illumina
184 platform. KneadData v0.7.10 (https://bitbucket.org/biobakery/kneaddata/) was then used for raw
185 sequence quality control and contaminant removal with default parameters. Short reads were
186 assembled into contigs using SPAdes v3.14.1[36] and each reference genome was annotated using
187 Prokka v1.14.6[37]. Additional annotation of antibiotic and antimicrobial resistance genes were
188 conducted using the Comprehensive Antibiotic Resistance Database (CARD)[38]. Assembled
189 contigs from All Purpose Probiotic Cleaner were also binned into five metagenome-assembled

190 genomes (MAGs) using metaWRAP[39]. CheckM[40] was used to assess the quality of microbial
191 genomes. Taxonomic classification of each draft genome was determined by GTDB-Tk[41].
192
193 *2.4. RNA Extraction, Sequencing, and Data Processing*
194
195 Swab samples were stored in RNAprotect Bacteria Reagent under -80°C until extraction. RNA
196 was extracted using the RNeasy Mini Kit (Qiagen) following its recommended "Protocol 2:
197 Enzymatic lysis and mechanical disruption of bacteria", consisting of a 10 min treatment with 15
198 mg/mL lysozyme and 10 min beadbeating. Libraries were constructed at Northwestern NUSeq
199 using the Illumina Total RNA Prep with Ribo-Zero Plus, according to manufacturers' protocol. A
200 total of 110 samples (108 samples collected at various time points and cleaning scenarios, one kit
201 control, and one sample prepared for between-batch normalization) were sequenced using an
202 Illumina HiSeq4000 with 50 bp single end reads.
203
204 Reference genomes constructed from whole genome sequencing were used for RNA mapping.
205 Raw sequence files were processed using trim_galore v0.6.6
206 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and Kneaddata v0.7.10
207 (https://bitbucket.org/biobakery/kneaddata/) to remove sequence adapters, low quality reads, and
208 contaminants. Sortmerna v4.2.0[42] was used to filter rRNA reads against the silva-bac-16s-id90
209 and silva-bac-23s-id98 databases. HISAT2 v2.1.0[43] was used to build local indices and map
210 short reads onto reference draft genomes. Transcript abundances were quantified using
211 featureCounts v2.0.1[44]. Differentially expressed genes (DEGs) were identified by DESeq2[45] at a
212 significance level of 0.05 (Benjamini-Hochberg adjusted p-value). BlastKOALA (KEGG

213 Orthology And Links Annotation) was used for K number assignment followed by Prokka
214 annotation[46]. KEGG pathway enrichment analysis was further conducted using ClusterProfiler[47].
215 Statistical analysis, including Welch's paired two sample *t*-test, principal component analysis
216 (PCA), and permutational multivariate analysis of variance (PERMANOVA, 9,999
217 permutations) were conducted in R (version 4.1.2, 2021-11-01).
218

219 ## 3. ***Results and Discussion***

220 ### ***3.1.*** *Survival of* A. baumannii *and* K. pneumoniae *under four cleaning scenarios and two*
221 *temperature/humidity conditions*

222

223 We quantified the CFUs of *A. baumannii* and *K. pneumoniae* to examine their survival under
224 ambient and humid conditions in combination with four cleaning strategies as listed in Table 1.
225 Over the course of 3 days, we observed a maximum $\log_{10}$ reduction of 8.75 for *A. baumannii*
226 (Cleaner Only and Probiotic Cleaner under ambient condition) and 7.42 for *K. pneumoniae* (No
227 Cleaner, Cleaner Only and Probiotic Cleaner groups). Under ambient conditions, both Cleaner
228 Only and Probiotic Cleaner demonstrated a near-complete inactivation of *A. baumannii* within
229 72 hours (<LOD) and complete inactivation for *K. pneumoniae*. Although the survival of *A.*
230 *baumannii* between Probiotic Cleaner and Cleaner Only are not statistically significant ($p>0.05$,
231 Figure S1), Probiotic Cleaner cleaning at 24 hours showed 1.43 greater $\log_{10}$ reduction compared
232 to Cleaner Only (Figure 2A). As there was no significant difference between probiotic cleaning
233 and cleaner only, the reduction of *A. baumannii* was largely attributed to the chemical
234 component of the cleaning product. Differences in the survival of *K. pneumoniae* between
235 Probiotic Cleaner, Cleaner Only and No Cleaning scenarios was not statistically significant

236 ($p>0.05$, Figure S1), with only 0.77 greater $\log_{10}$ reduction in Probiotic Cleaner compared to
237 Cleaner Only at 24 hours. The fact that *K. pneumoniae* was unable to persist on the surface in No
238 Cleaner group under ambient conditions indicated that chemical components in the cleaning
239 product only had marginal contribution to *K. pneumoniae* inactivation.
240
241 Surfaces cleaned with Probiotic Cleaner were also gradually dominated by *Bacillus* spp. due to
242 the decreasing absolute abundance of the pathogen population. This persistence of *Bacillus* spp.
243 on surfaces was observed under both ambient and elevated humidity and temperature (Figure 2).
244 In contrast, the germinated *Bacillus* spp. spores introduced as vegetative cells were gradually
245 taken over by the *A. baumannii* population; for *K. pneumoniae* Germinated Probiotic group the
246 cleaned surface was still dominated by *Bacillus* spp. over *K. pneumoniae* (Figure 2A). Without
247 the use of chemical-based surface cleaner (No Cleaner and Germinated *Bacillus*), *A. baumannii*
248 showed comparable CFUs at all time points ($p > 0.05$).
249
250 Throughout the experiments, the minimum $\log_{10}$ reduction was observed under elevated
251 temperature and humidity for both *A. baumannii* and *K. pneumoniae*. Their survival over time
252 improved under elevated temperature and humidity with or without probiotic cleaner (Figure 2)
253 although *A. baumannii* exhibited higher viable populations ($p<0.01$, Figure S1A) while *K.*
254 *pneumoniae* showed low or no significance (Figure S1B) when comparing No Cleaner (Humid)
255 to No Cleaner (Ambient) (paired *t*-test was performed for all time points). Higher viability of
256 bacteria is observed under higher RH, which is related to the stress caused by concentrating of
257 solute and desiccation as droplets dry out[48]. Genes *csrA* which is related to carbon storage and
258 *bfmR* (*rstA*) which is related to stress response play a major role in desiccation tolerance in *A.*

259 *baumannii* [49,50]. Both genes were downregulated under humid condition in *A. baumannii* No
260 Cleaner groups compared to ambient condition, and *csrA* gene was also downregulated under
261 humid condition in *K. pneumoniae* Probiotic Cleaner group compared to ambient condition ($\log_2$
262 fold change value < -1, adjusted p-value < 0.05, data not shown). This result suggests greater
263 potential health risks and infectious disease burdens in developing regions with a humid and hot
264 climate and limited climate control[51].

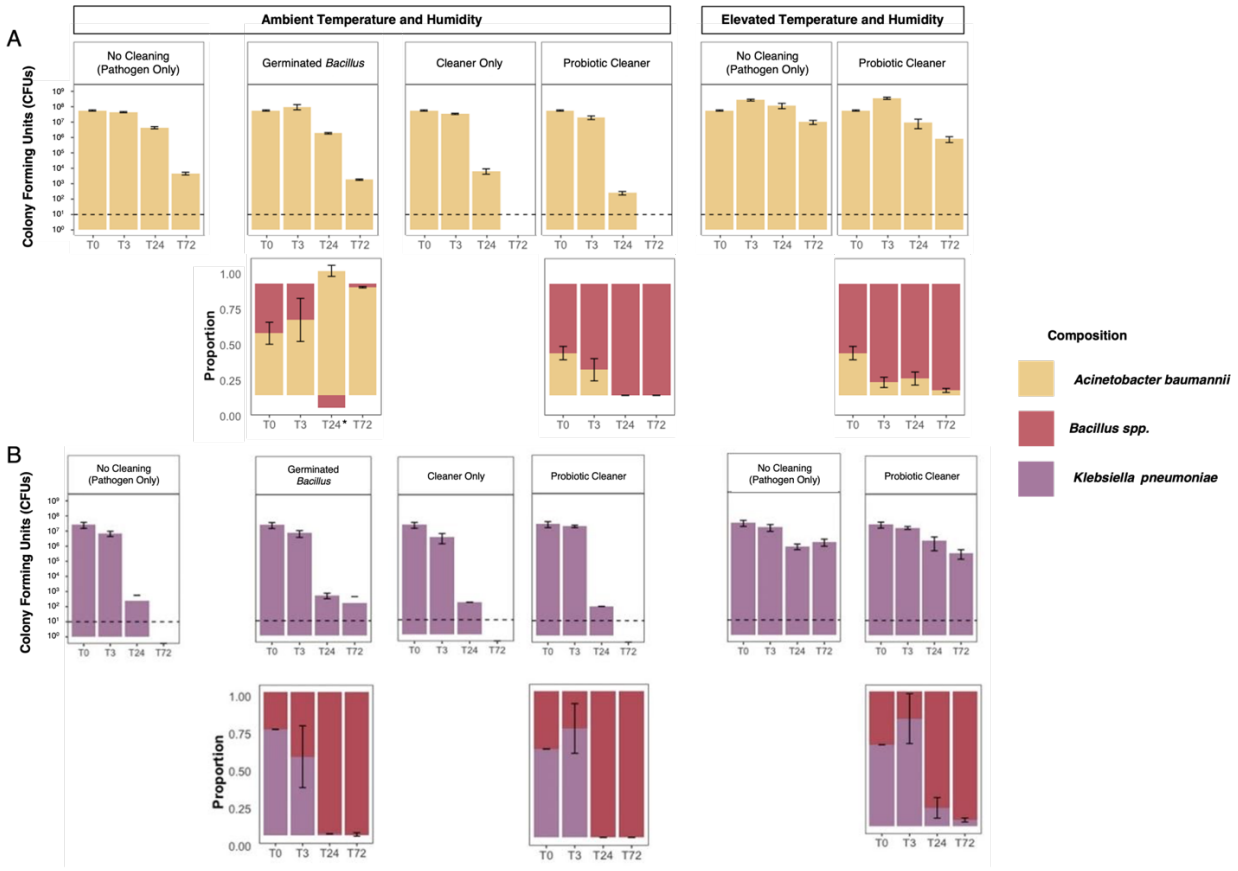


Figure 2. Colony forming units (CFU) of pathogens and composition of surface microbial community under four cleaning scenarios and two temperature and humidity conditions. CFUs of pathogens were counted on day 3 of incubation (37ºC) on their selective media while total CFUs (pathogen and *Bacillus* spp.) 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 $\mathbf{1} - \frac{\boldsymbol{CFU(CHROM\ or\ MacConkey)}}{\boldsymbol{CFU(TSA)}}$. 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 *A. baumannii* Germinated *Bacillus* scenario.

276 *3.2. Overview of whole genome sequencing and metatranscriptomic data*

277

278 Sequencing of the *A. baumannii* isolate generated 3.72 million reads with an average read length
279 of 136 bp. Reads were assembled into contigs with a N50 of 141,238 bp. Sequencing of *K.*
280 *pneumoniae* generated 4.4 million reads with an average rate of 145 bp. Reads were assembled
281 into contigs with a N50 of 113,698 bp. Sequencing of the All-Purpose Probiotic Cleaner resulted
282 in 16.49 million reads with an average read length of 139 bp, after quality trimming and
283 contaminant removal. Reads were assembled into contigs with a N50 of 143,313 bp. Open
284 reading frame (ORF) identification recognized 4,039, 5,823, and 22,429 coding regions for *A.*
285 *baumannii, K. pneumoniae,* and probiotic cleaner, respectively, 2,253, 4052, and 13,339 of
286 which were functionally annotated (not as "hypothetical protein"). Among these annotated
287 regions for *A. baumannii, K. pneumoniae,* and probiotic cleaner assemblies, 2,150, 3,675, and
288 11,594 were classified into one or more KEGG Orthologs respectively. Contigs from probiotic
289 cleaner reads were binned into five groups with completeness > 90%, and contamination < 5%.
290 All five bins were classified as *Bacillus* species as shown in Table 2.

291

292 **Table 2.** *Bacillus* genome binning and taxonomic assignment. Completeness and
293 contamination statistics were determined using the CheckM algorithm.

Bin	**Completeness (%)**	**Contamination (%)**	**GC (%)**	**Size (Mbp)**	**Taxonomic Classification**
1	90.58	1.883	0.351	4.74	*Bacillus cereus*

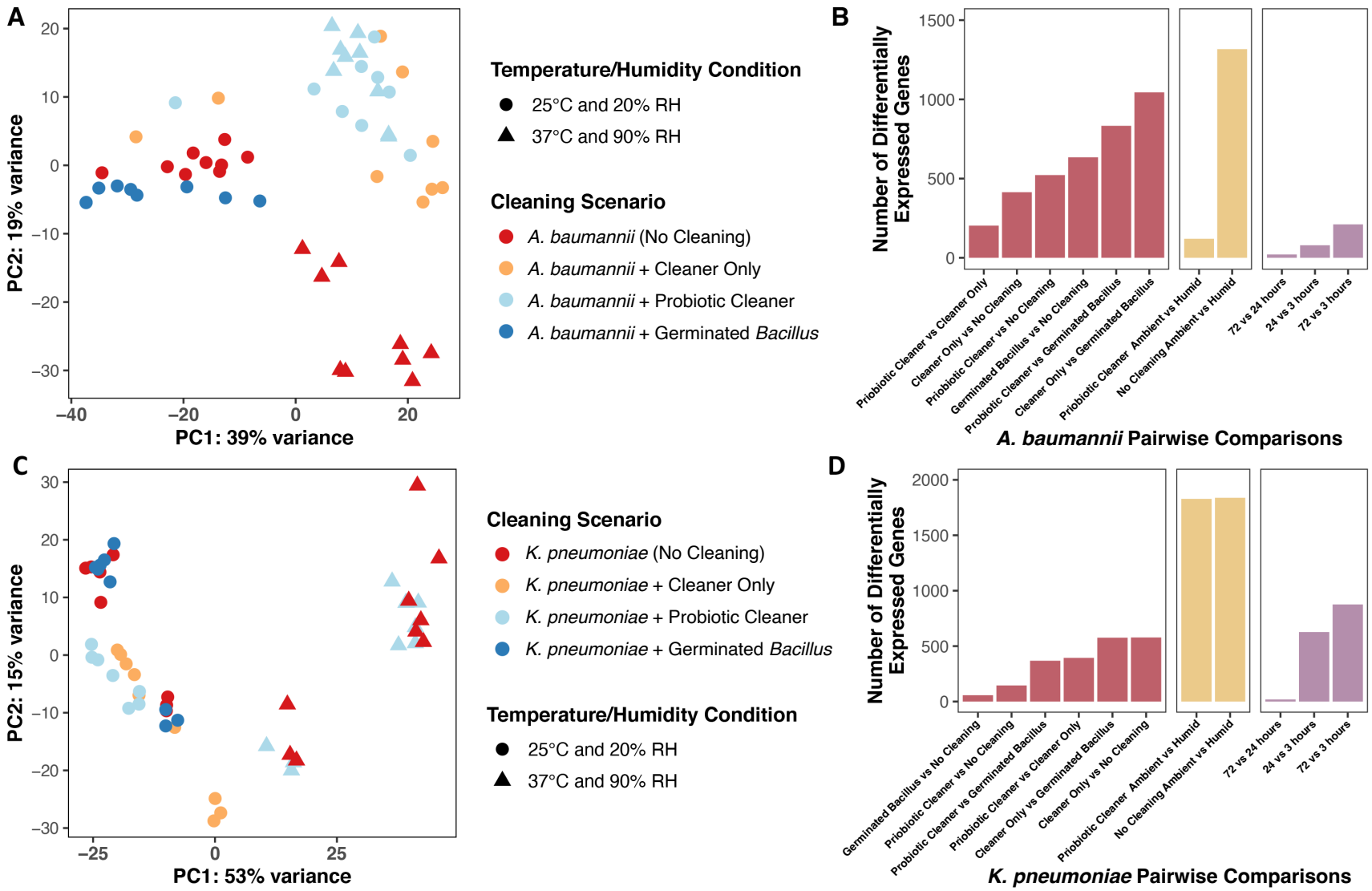
2	96.43	1.489	0.466	3.60	*Bacillus velezensis*
3	99.11	0.148	0.436	4.11	*Bacillus subtilis*
4	98.21	0.087	0.461	4.28	*Bacillus licheniformis*
5	99.08	0.207	0.416	3.57	*Bacillus safensis*

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; Germinated *Bacillus* samples containing vegetative *Bacillus* spp. on average had 40.53% ± 6.07% reads mapped onto *Bacillus* reference genomes, whereas this number for Probiotic Cleaner samples was only 0.56% ± 0.75%. A similar trend was observed in experiments with *K. pneumoniae*. Samples containing *K. pneumoniae* and 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 Probiotic Cleaner samples. Thus, these data suggest that the majority of *Bacillus* spp. in the probiotic product likely remained as spores throughout the incubation. Due to this insufficient coverage of *Bacillus* transcriptome in Probiotic Cleaner samples, our transcriptomic analysis is composed of quantitative comparisons

312 for the *A. baumannii* and *K. pneumoniae* transcriptomes and qualitative evaluations for *Bacillus*
313 spp.
314
315 3.3. *Transcriptomes of* A. baumannii *and* K. pneumoniae *clustered into distinct groups*
316 *determined by cleaning and physical conditions*
317
318 Prior to conducting principal component analysis (PCA), read count data were transformed to
319 remove the dependence of the variance on the mean. PCA of the transformed (variance
320 stabilization) read counts belonging to *A. baumannii* indicated that the expression of *A.*
321 *baumannii* clustered into three distinct groups based on cleaning scenarios and physical
322 conditions (Figure 3A). Clustering of read counts from *K. pneumoniae* is more distinct for
323 different time points and physical conditions (Figure S2) compared to cleaning scenarios and
324 physical conditions (Figure 3C). PERMANOVA (9,999 permutations) revealed that temperature
325 and humidity ($p<0.001$), cleaning ($p<0.001$), and time ($p<0.001$) explained 12%, 30%, and 9%
326 of the variance for *A. baumannii* read counts, and 29%, 10%, and 12% for *K. pneumoniae* read
327 counts, respectively. Gene expression profiles of *A. baumannii* with detergent cleaning (Probiotic
328 Cleaner and Cleaner Only) are more similar to each other than they were to those without
329 detergent (Germinated *Bacillus*). Temperature and humidity conditions also affected the
330 expression of *A. baumannii* under the no-cleaning condition, but this impact was minimal when
331 probiotic cleaner was used. On the contrary, the temperature and humidity strongly affected the
332 gene expression profiles of *K. pneumoniae*, and the impact was more pronounced as the cleaning
333 time extended from 3 hours to 24 and 72 hours.
334

335 Using No Cleaner samples as the reference transcriptome, genes with absolute $\log_2$ fold change
336 values greater than or equal to 1 as calculated by DESeq2 were considered as differentially
337 expressed genes (DEGs, Benjamini-Hochberg adjusted p-value ≤ 0.05). 414, 522, and 634 genes
338 of *A. baumannii* and 578, 145, and 57 genes of *K. pneumoniae* were found to be differentially
339 expressed in Cleaner Only, Probiotic Cleaner, and Germinated *Bacillus* samples, respectively.
340 The transcriptome of *A. baumannii* treated by probiotic cleaner and cleaner only shared the
341 highest level of similarity and consequently, the smallest number of differentially expressed
342 genes (Probiotic Cleaner *vs* Cleaner Only, Figure 3B). In the case of *K. pneumoniae*, Germinated
343 *Bacillus* samples were the most similar to No Cleaner samples. Consistent with previous PCA
344 analysis, expression of *A. baumannii* under ambient and elevated temperature and humidity
345 conditions are drastically different from each other (1298 DEGs, Figure 3B) whereas this
346 difference in transcriptome diminished when probiotic cleaner was used (133 DEGs). The high
347 impact of elevated temperature and humidity persisted for *K. pneumoniae* even when probiotic
348 cleaner was applied (1828 and 1838 DEGs with or without probiotic cleaner, Figure 3D). Since
349 the transcriptome of *K. pneumoniae* under Probiotic Cleaner scenario was fairly similar to the No
350 Cleaner scenario, their similar response to elevated temperature and humidity could be expected.
351 Samples collected at various time points throughout the microcosm inoculation also showed that
352 much of the temporal variation in gene expression occurred within 24 hours of surface cleaning
353 for *A. baumannii* (Figure 3B), *K. pneumoniae* (Figure 3D), and vegetative *Bacillus* (Figure S4),
354 as the number of DEGs of the 72-hour *vs*. 24-hour comparison is much lower compared to 24-
355 hour *vs*. 3-hour and 72-hour *vs*. 3-hour comparisons. During the experiment, we typically
356 observed drying out of the bacterial liquid culture droplets within 24 hours. This result is
357 consistent with study by Lin and Marr[52] on bacteria viability where bacteria inactivation rate is

358 higher in evaporating droplets compared to when droplets are dried out. Based on the
359 observation, cleaning protocols and environmental conditions should be considered for effective
360 disinfection in addition to cleaning reagent types.


A
PC2: 19% variance
PC1: 39% variance
Temperature/Humidity Condition
25°C and 20% RH
37°C and 90% RH
Cleaning Scenario
A. baumannii (No Cleaning)
A. baumannii + Cleaner Only
A. baumannii + Probiotic Cleaner
A. baumannii + Germinated Bacillus
B
Number of Differentially Expressed Genes
Priobiotic Cleaner vs Cleaner Only
Cleaner Only vs No Cleaning
Priobiotic Cleaner vs No Cleaning
Germinated Bacillus vs No Cleaning
Probiotic Cleaner vs Germinated Bacillus
Cleaner Only vs Germinated Bacillus
Priobiotic Cleaner Ambient vs Humid
No Cleaning Ambient vs Humid
72 vs 24 hours
24 vs 3 hours
72 vs 3 hours
A. baumannii Pairwise Comparisons
C
PC2: 15% variance
PC1: 53% variance
Cleaning Scenario
K. pneumoniae (No Cleaning)
K. pneumoniae + Cleaner Only
K. pneumoniae + Probiotic Cleaner
K. pneumoniae + Germinated Bacillus
Temperature/Humidity Condition
25°C and 20% RH
37°C and 90% RH
D
Number of Differentially Expressed Genes
Germinated Bacillus vs No Cleaning
Priobiotic Cleaner vs No Cleaning
Probiotic Cleaner vs Germinated Bacillus
Priobiotic Cleaner vs Cleaner Only
Cleaner Only vs Germinated Bacillus
Cleaner Only vs No Cleaning
Priobiotic Cleaner Ambient vs Humid
No Cleaning Ambient vs Humid
72 vs 24 hours
24 vs 3 hours
72 vs 3 hours
K. pneumoniae Pairwise Comparisons

361

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

366 *3.4. KEGG pathway enrichment profiles revealed different metabolic response under different*
367 *cleaning scenarios*
368
369 Enriched transcriptomic signatures varied greatly across cleaning conditions and between the
370 tested pathogens, *A. baumannii* and *K. pneumoniae*. Overall, the *K. pneumoniae* transcriptome
371 responded with greater heterogeneity to treatment than the *A. baumannii* transcriptome, as
372 evidenced by their distinct patterns of treatment-specific enrichment (Fig. 4A and 4D top
373 portion; e.g., *A. baumannii* with Germinated Probiotic). These phenomena support the hypothesis
374 that the *A. baumannii* genome encodes survival mechanisms for persistence in replete
375 environments, while *K. pneumoniae*, although capable of short-term response, may be less
376 equipped for long term survival on nutrient poor surfaces.
377
378 The *A. baumannii* transcriptome shifted under treatment conditions and 26 KEGG pathways
379 broadly related to metabolism, energy production, translation, and membrane transport were
380 enriched. Significant enrichment of translation machinery under Germinated Probiotic treatment,
381 suggests that protein synthesis in *A. baumannii* was likely promoted to achieve a large demand
382 for surface competitions. On the other hand, three pathways were positively enriched in samples
383 with chemical-based detergent cleaning, indicating that the *A. baumannii* population overall
384 repressed metabolic activities and adaptively reduced synthesis in response to environment
385 constraints associated with detergent cleaning (Figure 4A). As *A. baumannii* is notoriously
386 associated with the hospital environment[53], it stands to reason that it may remain constitutively
387 competent to respond to such replete environments and have acquired additional adaptive traits

388 to quickly acclimate when additional stressors, e.g., chemical cleaners, common in the hospital
389 environment arise.
390
391 In contrast, *K. pneumoniae* responded with far greater heterogeneity at the transcript level.
392 Carbon metabolism genes in *K. pneumoniae* were mutually upregulated in every cleaning
393 condition; however, specific carbon metabolism pathways were induced in a treatment-
394 dependent manner. For instance, TCA cycle, pyruvate, starch and sucrose metabolism genes
395 were upregulated by treatment with germinated *Bacillus* or cleaner only but not in samples
396 treated with probiotic cleaner. This suggests that vegetative *Bacillus* and cleaning products
397 induce alternative though overlapping carbon metabolism networks in *K. pneumoniae*. Indeed,
398 increased expression of biosynthetic pathways (glyoxylate cycle, carbon fixation) suggests
399 vegetative *Bacillus* may promote anabolic competency in *K. pneumoniae*. Perhaps this broad
400 range of metabolic activity is the result of slight alterations in the *K. pneumoniae* panic response,
401 as the population tries to survive in a "hostile" environment, unlike that of its native host.
402
403 While fewer pathways (11) were uniquely enriched in response to germinated *Bacillus,* every
404 treatment stimulated some unique pathway upregulation in *K. pneumoniae,* unlike the uniform
405 pattern observed in *A. baumannii* (Fig 4D). Genes involved in two-component systems and
406 membrane transport (ABC) were upregulated in samples treated with probiotic cleaner or cleaner
407 only but not in samples containing vegetative *Bacillus*, indicating that chemical detergents, rather
408 than *Bacillus*, induce environmental sensing and response mechanisms in *K. pneumoniae*.
409 *Surprisingly*, no pathways were mutually and specifically enriched by vegetative *Bacillus* or
410 probiotic cleaner, which may be influenced by the diminished transcriptional landscape of

411 *Bacillus* spores (Figure S2). Alternatively, the divergent transcriptomic profiles induced by each
412 cleaning condition indicates that *K. pneumoniae*'s strategy for surviving on surface environments
413 vastly differs from *A. baumanii* and further suggests that the *A. baumanii* response system to
414 built environment surfaces may be more robust than *K. pneumoniae*'s. Indeed, evolutionary
415 studies modelling extended resource exhaustion in *Escherichia coli* have shown that while stress
416 responses dominate in the short-term, in the long-term, genetically-encoded adaptations to
417 replete environments sweep the population via clonal expansion[54,55]. One could thus hypothesize
418 the dissimilar fitness levels and phenotypic responses (e.g., enrichment patterns) of *K.*
419 *pneumoniae*[56] and *A. baumanii* in this study may be the results of their unique evolutionary
420 history and ecological niches.
421
422 Cleaning products may represent a source of nutrients in an otherwise replete environment. Both
423 *A. baumannii* and *K. pneumoniae* exhibited increased transcription of genes related to
424 metabolism, although not the same pathways and not under all conditions. Our results suggest
425 that vegetative *Bacillus* survive poorly on surfaces and become a source of nutrients to more
426 resilient *A. baumannii*. *K. pneumoniae* enhances transcription of various metabolic pathways, but
427 none of the responses enhance its survival. The specific metabolic activity of organisms on
428 surfaces is highly dependent on both the organism and the resource availability.

429

430 *3.5. Different cleaning scenarios induced differential expression of genes associated with stress*
431 *response and competition*

432

433 We examined gene expression of important interspecies competition pathways to understand the
434 potential interactions between probiotic bacteria *Bacillus* spp. and pathogens *A. baumannii* or *K.*
435 *pneumoniae*. The two pathogens responded differently toward cleaning scenarios where *A.*
436 *baumannii* exhibited more pronounced differential gene expression compared to *K. pneumoniae*.

437

438 Iron acquisition through siderophores is believed to be one of the key mechanisms of inter-
439 species competitive exclusion[57]. Differential expression of the siderophore and iron acquisition
440 genes indicated that siderophore cheating is unlikely to happen under our studied conditions. In
441 *A. baumannii* experiments, several genes responsible for siderophore synthesis, export, and
442 reception (e.g., aerobactin, anguibactin, and enterobactin) were downregulated in the Germinated
443 Probiotic group (Figure S5A). Although bacteria with multiple siderophore receptors can gain
444 competitive advantages in social competition through siderophore cheating[58], the downregulation
445 of siderophore-related genes in these samples was likely due to utilization of iron originating
446 from lysed cell debris, especially from *Bacillus* spp., rather than active competition through
447 siderophore cheating, given the inability of vegetative *Bacillus* spp. to persist on surfaces (Figure
448 2A). For *K. pneumoniae* experiments, no siderophore-related gene was differentially expressed
449 under the three cleaning scenarios (Figure S6A, *iutA*, *fepA*, and *fepC* $\log_2$ fold change absolute
450 values < 1). Gene expression levels of *Bacillus* spp. (Figure S7) further precluded siderophore
451 cheating as the competing mechanism on surface as genes related to the synthesis, and export of

452 *Acinetobacter*- and *Klebsiella*-associated siderophores (e.g., aerobactin, anguibactin, and
453 enterobactin) were only occasionally expressed (Figure S7).
454
455 In addition to iron acquisition, genes related to Type VI Secretion Systems (T6SS), Type IV or
456 Type 1 Pili, and biofilm formation were shown to have important implications in inter-species
457 competition, virulence expression, and starvation response in *A. baumannii* or *K. pneumoniae*
458 [57,59,60]. In *A. baumannii* experiments, genes associated with T6SS, Type IV Pili, poly-beta-1,6-N-
459 acetyl-D-glucosamine (PGA) production and biofim formation were upregulated in Probiotic
460 Cleaner, Cleaner Only, and Germinated Probiotic samples with varying statistical significance
461 (Figure S5). The *pgaABCD* operons, which had increased expression in Germinated Probiotic
462 group, play roles in surface binding and maintaining biofilm structure stability[61,62]. This result
463 suggested greater potential for PGA, a biofilm adhesin polysaccharide[61,62], production and
464 subsequent biofilm activities when *A. baumannii* and *Bacillus* spp. co-existed. In contrast, *K.*
465 *pneumoniae* exhibited negligible differences in expression levels for genes related to T6SS and
466 Type 1 Pili had under different cleaning scenarios for (Figure S6BD). Additionally, only a few
467 biofilm formation related genes were differentially expressed (Figure S7C). Lack of shared
468 differentially expressed genes across cleaning scenarios indicated nonspecific response of *K.*
469 *pneumoniae* biofilm formation towards different stressors.
470
471 Similar to what was observed for KEGG pathways, antibiotic resistance gene expression of
472 probiotic cleaner and cleaner only treated *A. baumannii* exhibited high similarity (Figure 4B)
473 while *K. pneumoniae* responded very differently to probiotic cleaner and clear only treatments
474 (Figure 4E) in. In the Germinated Probiotic groups, we observed an increased expression of

475 *abaF*, a gene encoding fosfomycin resistance, in *A. baumannii* while no antibiotic resistance
476 gene was upregulated ($\log_2$ fold change ≥ 1) in *K. pneumoniae*. Interestingly, no genetic
477 determinants of fosfomycin biosynthesis were identified from *Bacillus* spp. reference genomes.
478 In addition, it was reported that the disruption of *abaF* in *A. baumannii* not only resulted in an
479 increase in fosfomycin susceptibility but also a decrease in biofilm formation and virulence[63].
480 Therefore, the upregulation of *abaF* in *A. baumannii* may not be induced by the fosfomycin
481 secretion from *Bacillus* spp. but rather a stress response to *Bacillus* spp. existence exerting
482 enhanced biofilm formation.
483
484 In *A. baumannii* experiments, pronounced downregulation of genes related to sulfur metabolism,
485 particularly those induced by sulfur starvation, was observed for groups where chemical cleaning
486 detergent was present (Probiotic Cleaner and Cleaner Only groups for the ambient condition,
487 Figure 4C; Probiotic Cleaner group for the humid condition, Figure 5C). These genes include
488 *ssuEADCB* and *tauABCD* operons known to assimilate sulfur from aliphatic sulfonates[64] and
489 facilitate the utilization of taurine as an alternative sulfur source under sulfate-deprived
490 environment[65]. Owing to the presence of anionic surfactants in liquid detergent products, such as
491 sodium lauryl ether sulfate (SLES)[66], these sulfate starvation genes were likely suppressed in
492 Cleaner Only and Probiotic Cleaner groups. This observation is consistent with studies that
493 illustrated the overexpression of *tauABCD* and *ssuEADCB* genes induced by sulfate shortage[67].
494 Additionally, bacterial utilization of SLES facilitated by *Citrobacter braakii* [68] and a consortium
495 of *Acinetobacter calcoacetiacus*, *Klebsiella oxytoca*, and *Serratia odorifera*[66] has also been
496 reported in previous studies and may contribute to the persistence of microbial communities
497 associated with hospital sinks[69]. On the contrary, no differential expression was observed for

498 sulfur metabolism genes in *K. pneumoniae* Probiotic Cleaner group under both ambient and
499 humid conditions (Figure 4F and Figure 5F).
500
501 In *K. pneumoniae* experiments, Cleaner Only and Germinated Probiotic groups showed
502 similarities in gene expressions while Probiotic Cleaner appeared to have very limited effects on
503 *K. pneumoniae* gene expression. It is possible that the dormant *Bacillus* spores in probiotic
504 cleaner were buffering the stress caused by chemical cleaner while the vegetative *Bacillus* cells
505 introduced competition pressure on the surfaces. Thus, it is important to understand how spores
506 and vegetative probiotic microorganisms interact with and affect the performance of chemical
507 surface cleaning reagents, which can alter the response landscape of the surface associated
508 pathogens. Since the results from our study showed species-specific responses, more pathogen
509 species should be investigated for their interspecies interactions with probiotic microorganisms
510 to observe general patterns. This will set the foundation and facilitate the development of
511 targeted cleaning and disinfection strategies.

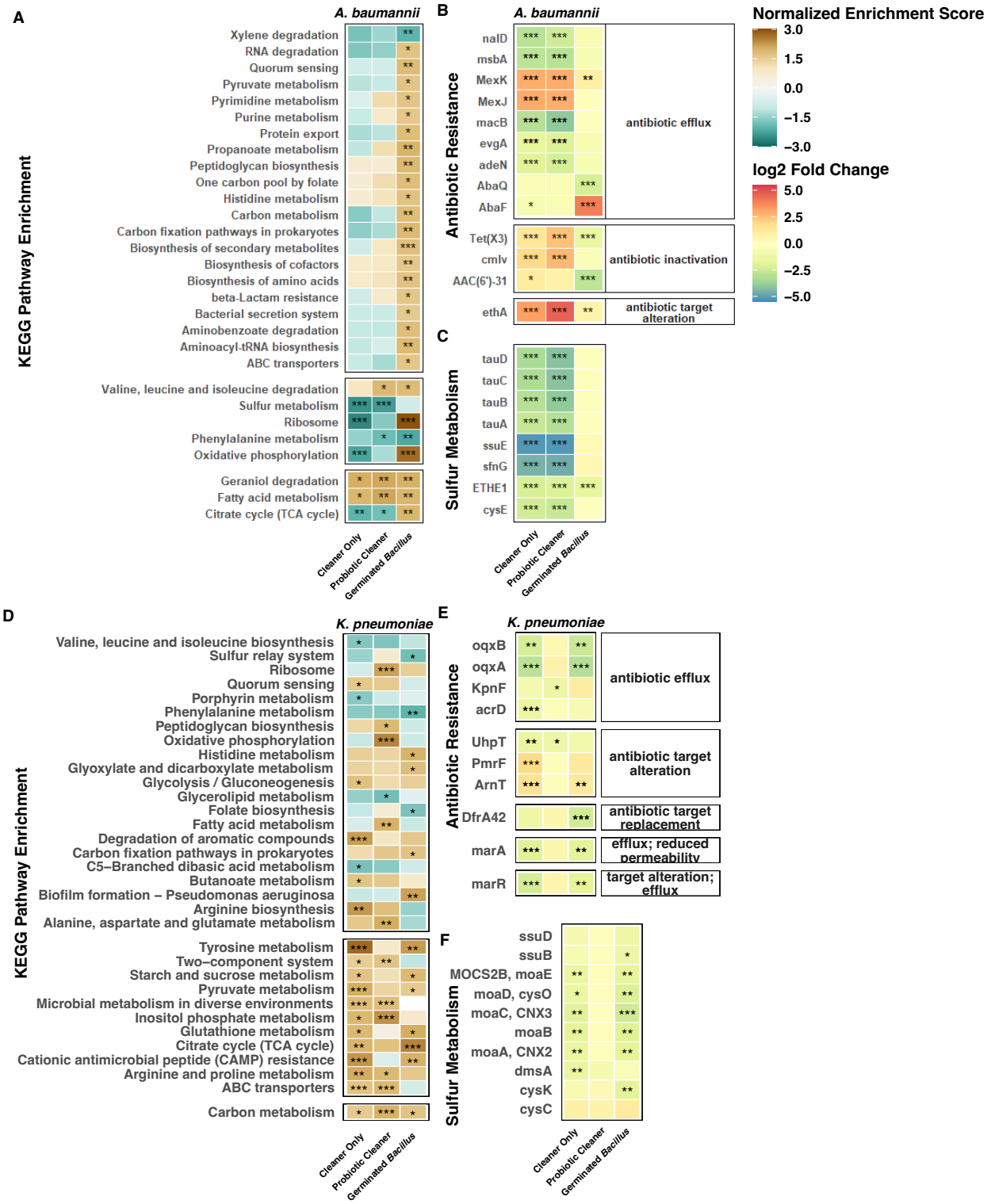
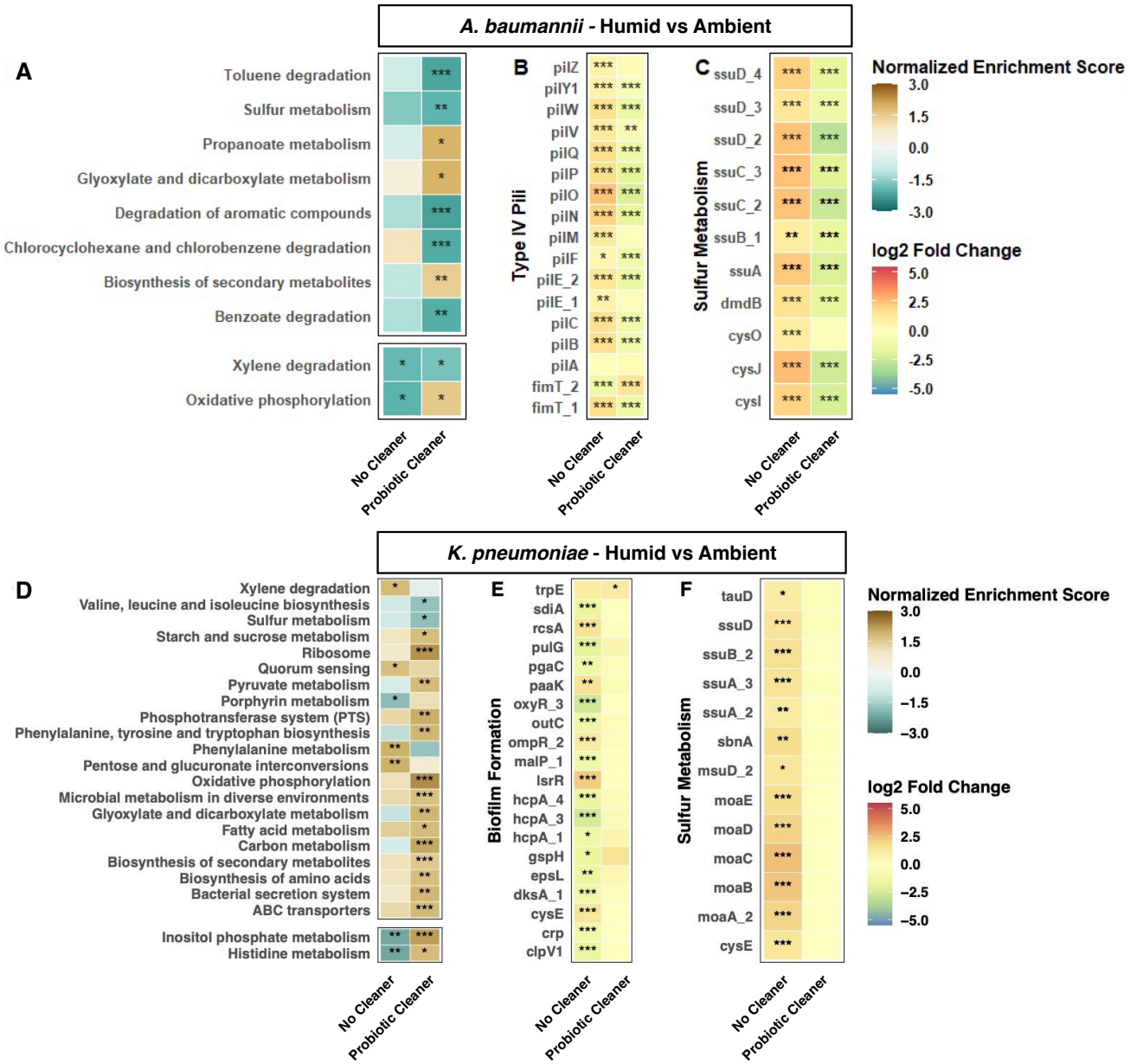


Figure 4. KEGG pathway enrichment results for samples collected under ambient temperature and humidity condition (A, D), $\log_2$ fold change of genes associated with

515 antibiotic/antimicrobial resistance (B, E), and sulfur metabolism (C, F), compared to no-
516 cleaning samples (*A. baumannii* or *K. pneumoniae*). KEGG pathways shown here were
517 statistically enriched in at least one of the cleaning conditions (Benjamini-Hochberg
518 corrected p-value ≤ 0.05). Normalized enrichment score (NES) accounts for differences
519 in gene set size and were used to compare enrichment results across KEGG pathways;
520 a positive NES suggests an overall positive upregulation of genes belonging to its
521 corresponding pathway[47,70,71]. * $p<0.05$, ** $p< 0.01$, and *** $p < 0.001$.



522

523 **Figure 5.** KEGG pathway enrichment results for No Cleaner and Probiotic Cleaner

524 samples collected under elevated temperature and humidity condition (A, D), $\log_2$ fold

525 change of genes associated with *A. baumannii* cell motility (B), *K. pneumonia*e biofilm

526 formation (E), and sulfur metabolism (C, F). KEGG pathways shown here were

527 statistically enriched in at least one of the cleaning conditions (Benjamini-Hochberg

528 corrected p-value ≤ 0.05). * p<0.05, ** p< 0.01, ***p < 0.001.

529 3.6. *Effects of elevated temperature and humidity on pathogen cell motility and biofilm formation*

530

531 Although over 1,000 differentially expressed genes were identified from *A. baumannii* or *K.*
532 *pneumoniae* transcriptomes between the ambient and humid conditions, the majority of the
533 KEGG pathways were composed of genes that were both upregulated and downregulated,
534 rendering non-significant results for most pathways.

535

536 Under the humid condition (elevated temperature and humidity), 16 KEGG pathways related to
537 metabolism (10), membrane transport (3), biosynthesis (2) and translation (1) were enriched in
538 *K. pneumoniae* experiments (Figure 5D, Probiotic Cleaner Humid *vs* No Cleaner Humid) while
539 only 4 KEGG pathways related to metabolism (2), biosynthesis (1) and energy production (1)
540 were enriched in *A. baumannii* (Figure 5A, Probiotic Cleaner Humid *vs A. baumannii* Humid)
541 with Probiotic Cleaner. Overall, more KEGG pathways were affected by elevated temperature
542 and humidity in *K. pneumoniae* than *A. baumannii* (Figure 5D and Figure 5A). This result
543 corresponded to the PCA and PERMANOVA results where temperature and humidity affected
544 the transcriptome profiles of *K. pneumoniae* more than *A. baumannii*, but not reflected in the
545 survival of the two pathogens under elevated temperature and humidity assessed by viable CFU
546 counts.

547

548 Without Probiotic Cleaner, cell motility appeared to be affected differently by increased moisture
549 availability for *A. baumannii* and *K. pneumoniae*. *A. baumannii* exhibited upregulation of type
550 IV pili genes (Figure 5B) whereas *K. pneumoniae* type 1 pili genes remained unaffected, which
551 was also the case under ambient condition (Figure S6D). Nevertheless, 19 genes related to

552 biofilm formation were differentially expressed (Figure 5E, *K. pneumoniae* Humid vs *K.*
553 *pneumoniae* Ambient), and the quorum sensing pathway, which affects biofilm formation of *K.*
554 *pneumoniae*[72] , was enriched under elevated temperature and humidity (Figure 5D, *K.*
555 *pneumoniae* Humid vs *K. pneumoniae* Ambient). This indicated that elevated temperature and
556 humidity could assist the persistence of *K. pneumoniae* on surfaces (Figure 2B) by altering the
557 biofilm formation related gene expression.
558
559 For both *A. baumannii* and *K. pneumoniae* under Probiotic Cleaner scenario, sulfur metabolism
560 pathway was downregulated under elevated temperature and humidity, which is consistent with
561 the sulfur metabolism-related gene expression results discussed in the previous section when
562 chemical detergent was present. The effects of probiotic cleaner on *K. pneumoniae* were likely
563 masked by the changes due to higher temperature and humidity (Figure 5E and Figure 5F),
564 meaning the benefits of probiotic cleaning products could be more limited in tropical areas for
565 certain types of pathogens.
566
567 Our results demonstrated the persistence of probiotic *Bacillus* included in the All-Purpose
568 Probiotic Cleaner up to 72 hours after cleaning. However, this persistence did not result in more
569 efficient reduction of pathogen biomass. *A. baumannii* on surfaces cleaned using chemical-based
570 detergent with and without probiotic *Bacillus* contained a comparable amount of viable
571 pathogens. The transcriptome of *A. baumannii* with and without probiotic addition shared a high
572 degree of similarity in overall gene expression. In contrast, the transcriptome of *K. pneumoniae*
573 with probiotic addition showed a high degree of differences compared to samples with chemical
574 cleaner only or germinated *Bacillus*, including reduced response in genes related to antimicrobial

575 resistance, sulfur metabolism and biofilm formation. We thus conclude *Bacillus* spores indeed
576 survive unfavorable conditions, but that the small percentage of germinated *Bacillus* cells and/or
577 slow germination rate on surfaces preclude interspecies interactions like competitive exclusion.
578 Elevated temperature and humidity resulted in prolonged persistence of viable pathogens on
579 surfaces. These conditions also had a pronounced effect on *K. pneumoniae* overall gene
580 expression regardless of which cleaner was used. These results suggest that vigilant indoor
581 climate control could contribute to infection prevention. Especially in tropical environments, our
582 results suggest that reducing the temperature and relative humidity is a more important strategy
583 for infection prevention than cleaning product selection.
584
585 The design of more effective probiotic cleaning products and strategies to promote "healthy"
586 microorganisms will require a more nuanced understanding of the relevant environmental
587 conditions and how they impact activity. Our study used microcosms to model environmental
588 conditions *in situ* and enable the manipulation of individual parameters. While our microcosms
589 do not represent the life histories of organisms *in situ* (e.g., previous exposure and selection on a
590 surface), this type of work forms an important bridge between experiments in culture media,
591 which do not reflect *in situ* conditions, and field studies, which preclude replication and
592 manipulation. We further demonstrate that transcriptomics can be used to assess activity on
593 surfaces. We advocate for the inclusion of such assessments in field studies, which are
594 historically typically DNA- or culture-based, especially as RNA sequencing methods improve
595 for low biomass samples. The assessment of cleaning and disinfection is currently based on log
596 reductions of viable biomass. Using these tools to progress beyond taxonomic and viability

597 profiling will allow us to include more nuanced endpoints, such as the prevention of difficult-to-
598 disinfect phenotypes like biofilm formation or antimicrobial resistance.
599
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610
611 ***Competing Interests***
612
613 The Authors declare no Competing Financial or Non-Financial Interests.
614
615 ***Author Contributions***
616
617 JH designed and performed the experiments, analyzed data, and wrote the manuscript. WS, JTS,
618 and AAM analyzed transcriptomics data and wrote the manuscript. EMH supervised the project.
619

620 ***Data Availability***

621

622 Raw sequencing data are available in NCBI Sequence Read Archive (SRA) under BioProject

623 accession number PRJNA818256. Processed data files and scripts for data analysis are available

624 in GitHub at https://github.com/Swt623/Probiotic_competition.git.

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626

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