A Novel Technique to Characterize *Klebsiella pneumoniae* Populations Indicates that Mono-Colonization is Associated with Risk of Infection

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Abstract

Klebsiella pneumoniae and related species are a common cause of healthcare-associated infections. The gut is a major *Klebsiella* reservoir and gut colonization is a risk factor for developing an extraintestinal *Klebsiella* infection. Patients can be colonized by multiple *Klebsiella* strains or even species in the gut simultaneously, and there is high concordance between the gut colonizing- and infection causing- strains. The detection and characterization of colonizing strains is critical for a better understanding of the progression to infection and for developing interventions for colonized patients. However, the association between mixed or mono-colonization and subsequent infection is unknown. In this study, we developed an amplicon-based sequencing method called *wzi*-Seq that enables the detection and quantification of *Klebsiella* strains from complex samples and mixtures using the conserved capsule gene *wzi* as a molecular barcode. This method is highly accurate and precise with a sensitivity of 93% and specificity of 99.8% in mixtures containing as many as 58 unique *wzi* types. The assay was validated analytically and applied to an established case and control cohort. We determined that 63.2% (108/171) patients were mono-colonized with a single *Klebsiella* strain while 36.8% (63/171) had mixed colonization with multiple *Klebsiella* strains. Controlling for patient variables in multivariate analysis, we determined that mono-colonization was significantly ($p = 0.034$) associated with infection. Characterization of *Klebsiella* colonizing populations could improve the accuracy of assessing infection risk and enable targeted interventions to prevent these healthcare-associated infections.

Importance

Klebsiella gut colonization is a major risk factor the development of extraintestinal *Klebsiella* infections in hospitalized settings. However, it is unknown if patients are colonized by one or multiple strains of *Klebsiella* and how the population structure of *Klebsiella* in the gut impacts infection risk. Here we describe the development of a novel technique, *wzi*-Seq, to detect and

quantify multiple *Klebsiella* strains from complex samples using standard techniques and rapid DNA sequencing. We applied *wzi*-Seq to rectal swabs from a well-characterized patient cohort and found that *Klebsiella* colonization with a single strain was more prevalent than colonization with multiple strains. Furthermore, mono-colonized patients had a significantly higher risk of developing a *Klebsiella* infection than mixed colonized patients. This work validates a new tool to study *Klebsiella* populations and reveals that population structure in the gut influences the risk of healthcare associated infections.

Introduction

The Gram-negative bacterium *Klebsiella pneumoniae* (*Kp*) has become a pathogen of major concern due to increasing virulence, infections, and antibiotic resistance[1,2]. The rise in *Kp* antibiotic resistance is especially concerning as *Kp* has become the third highest cause of deaths attributable or associated to antimicrobial resistance globally[3]. *Kp* is part of a species complex (KpSC) of five closely related species and their subspecies: *K. pneumoniae*, *K. variicola, K. quasipneumoniae*, *K. quasivariicola*, and *K. africana*[4]. These species can colonize mucosal surfaces in the body such as the nasopharynx and gut and can cause pneumonia, urinary tract infections, and bacteremia[5,6].

One of the primary *Kp* reservoirs is the gut with asymptomatic gut colonization rates typically ranging between 6-23% [7–10]. *Kp* gut colonization is a major risk factor for the development of an extraintestinal *Kp* infection[11,12]. In approximately 80% of patients there is concordance between the gut colonizing and infection causing strain[8,13]. Colonization data is based typically on a few cultured rectal isolates per patient, preventing a clear understanding of colonizing *Kp* dynamics in patients. Even with this limited sampling, we occasionally observed that patients were colonized with more than one strain simultaneously[13]. The true incidence of mixed colonization

71 in patients, and its impact on infection risk is a major gap in knowledge. For example, mixed
72 colonization could represent a low-risk state in which commensalism between strains, and the
73 larger community prevents progression to disease, or an extremely dysbiotic state where niche
74 exclusion is compromised and multiple pathogenic strains of *Kp* invade and increase the risk of
75 infection.

76

77 Molecular typing schemes to identify and track *Kp* strains, including multilocus sequence typing
78 (MLST), pulsed-field gel electrophoresis (PFGE), whole genome sequencing (WGS), and
79 capsule typing are highly standardized and reproducible but are often time and labor intensive[14–
80 18]. In recent years, strain typing based on the conserved *wzi* gene in the *Kp* capsule operon has
81 become an alternative method for distinguishing *Kp* strains[16]. Previous work has validated *wzi*
82 primers for the conserved motifs that flank the highly variable 447 bp region of the *wzi* gene
83 (Fig. 1)[16]. Sanger sequencing of the *Kp wzi* gene using these primers has enabled the curation
84 of the BigsDB database (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html) containing 741
85 unique *wzi* types[16,19]. Strain identification via *wzi* typing has been shown to have higher
86 discriminatory power than traditional K typing and similar discriminatory power as MLST
87 typing[16]. This technique has become a fast and reliable alternative method for the differentiation
88 of *Kp* strains.

89

90 To better characterize *Kp* colonization dynamics and the impact on patient infection risk we
91 adapted *wzi* sequencing primers to develop a high throughput and accurate amplicon-based
92 method called *wzi*-Seq[16]. The goal of this study was to develop and validate the *wzi*-Seq
93 method, use it to assess the incidence of mono- vs mixed colonization in patients, and evaluate
94 the impact of colonization status on patient infection risk. We demonstrated that *wzi*-Seq is a

95 highly accurate and precise method with a sensitivity of 93% and specificity of 99.8% for
96 detecting strains in complex mixtures containing up to 58 *wzi* types. We applied *wzi*-Seq to
97 patient rectal swabs from a previous case-control study and demonstrated that mono-*Kp*
98 colonization was more prevalent than mixed *Kp* colonization and associated with an increased
99 risk of developing a subsequent extraintestinal *Kp* infection[13,20]. This work describes a novel
100 method of using the *wzi* gene as a molecular barcode and provides insight into *Kp* colonization
101 dynamics that has the potential to improve our understanding of *Kp* colonization dynamics and
102 patient infection risk.

103 **Results**

104 *wzi-Seq Assay Design*

105 To design an amplicon-based sequencing method for the detection and quantification of multiple
106 *Kp* strains from complex samples, we developed a nanopore-based long-read amplicon
107 sequencing technique targeting the conserved *wzi* gene (Fig. 1). Sequencing reads were
108 mapped to a reference database of 741 unique *wzi* alleles, and two quality filtering pipelines
109 were used, which differed in allowing no mismatches (error0) or an error rate of up to 0.5% of
110 the alignment length (error0.005). Whereas the error0 pipeline was more stringent for the
111 presence of a strain, the error0.005 pipeline was expected to produce more reads for
112 quantification of abundance. After quality filtering, the remaining reads for each *wzi* type were
113 used to calculate the total abundance of each *wzi* type in a sample.

114

115 *Accuracy and Precision*

116 To validate the *wzi*-Seq approach, contrived samples were created using increasingly complex
117 mixtures of strains with known *wzi* types. *Kp* strains with unique *wzi* types were mixed at known
118 ratios to create 1, 5,12, 30 or 58 *wzi* type sample mixtures. Sequencing of a single *Kp* strain

119 KPPR1 (*wzi* type 2), yielded 13,367 and 6,181 reads from the error0.005 and error0 pipelines,
120 respectively. Of these reads, the total correct mapped reads were 13,338 and 6,179 for the
121 error0.005 and error0 pipelines, with a read accuracy greater than 99.7% for both pipelines
122 (Suppl. Table 1). Although both pipelines had reads that mapped to at least two additional *wzi*
123 types that were not the expected *wzi* type, these reads were less than 0.3% of the total for
124 either pipeline (Suppl. Table 1).

125

126 To further test the accuracy and precision of the *wzi*-Seq method, a 5 *wzi* type mixture was
127 created using *Kp* strains with *wzi* types 2, 57, 19, 522, 322. The *wzi* types were mixed using
128 intended ratios of 50%, 30%, 15%, 4%, and 1% of each strain based on optical density (OD)
129 measurements. An average of 9,932 and 4,322 reads were recovered from the error0.005 and
130 error0 pipelines, respectively with a read accuracy greater than 99% for both pipelines (Suppl.
131 Table 2). For the error0.005 pipeline, the mean *wzi* type abundance was within 1% of the
132 expected abundance and there was high precision between replicates (standard deviation <1%
133 for each *wzi* type; Table 1). Although the error0 pipeline had higher total read accuracy, the
134 pipeline was slightly less precise (standard deviation: 0.1-1.1%) compared to the error0.005
135 pipeline (standard deviation: 0.1-0.6%) (Table 1). When comparing the experimental abundance
136 values between the two pipelines there were no significant differences in the abundance values
137 for each *wzi* type (Fig. 2A).

138

139 Next, we tested more complex mixtures containing between 12-58 unique *wzi* types.
140 Sequencing a 12 *wzi* type mixture in triplicate yielded 96,296 and 47,064 correctly mapped
141 reads for the error0.005 and error0 pipelines, respectively (Suppl. Table 2), for a read accuracy
142 greater than 99%. There was no significant difference in the recovered abundance values for

143 each *wzi* type between the two pipelines (Fig. 2B). Similarly to the 5 *wzi* type mixture, the
144 precision between replicates was high with a standard deviation of less than 2% for the
145 abundance of any of the 12 *wzi* types (Suppl. Table 3). Although more than 12 different *wzi*
146 types were recovered by both pipelines, these erroneous reads made up less than 0.5% of all
147 total reads and there was no significant difference in the abundance of each *wzi* type between
148 the two pipelines (Fig. 2B, Suppl. Table 2). Additionally, the *wzi*-Seq method was tested in
149 duplicate on 30 and 58 *wzi* type mixtures (Suppl. Table 4). The read accuracy was greater than
150 99% for the error0 pipeline, and 95.5% and 97.0% for the 30 and 58 *wzi* type mixtures,
151 respectively, with the error0.005 pipeline (Suppl. Table 4). Together these data highlight that *wzi*-
152 Seq can accurately differentiate *wzi* types in complex mixtures containing up to 58 unique *wzi*
153 types.

154

155 To enable analysis of low-biomass samples in which *Kp* may be present in low abundance, the
156 accuracy and precision of performing a second round of PCR was tested on 5 and 12 *wzi* type
157 mixtures. There were no significant differences in the abundance values after one or two rounds
158 of PCR in the 12 *wzi* type mixture regardless of whether the error0.005 or error0 pipeline was
159 used (Fig. 2C, D). No differences between the number of rounds PCR and abundance levels
160 were also observed for the 5 *wzi* type sample mixture (Table 1). Together these data highlight
161 the high accuracy and precision of the *wzi*-Seq method and that this accuracy and precision is
162 maintained with increasingly complex mixtures and low biomass samples.

163

164 *Sensitivity and Specificity of Strain Detection*

165 Next, we assessed the true positive and false positive rates of strain detection in contrived
166 mixtures. The *wzi* abundance values of 23 different contrived samples with mixtures ranging in

167 complexity from 5-58 *wzi* types were used as the input to generate a receiver operating
168 characteristic (ROC) curve. All detected *wzi* types that were expected in each sample were
169 considered true positive values and all other *wzi* types were considered false positives. For the
170 error0.005 and error0 pipelines, the area under the ROC was 0.9925 and 0.9986, indicating
171 very high accuracy (Fig. 3A, B). The ROC curves were used to determine cutoffs for both
172 pipelines that would optimize sensitivity and specificity of strain detection at the readfrac level
173 before converting to relative abundance. When using a readfrac cutoff of 0.00275 on error0.005
174 samples, the predicted sensitivity and specificity of the assay was 93.0% and 99.8%,
175 respectively (Fig. 3C). For the error0 pipeline, a readfrac cutoff of 0.00172 predicted an assay
176 sensitivity and specificity of 94.2% and 100%, respectively (Fig. 3C). These cutoffs were then
177 tested on four contrived mixtures containing between 5-30 different *wzi* types that were not
178 included in the original ROC analysis. Applying these cutoffs eliminated 98.9% of false positives
179 in error0.005 samples and 100% of false positives in error0 samples with 100% of true positives
180 detected in all samples (data not shown) and were applied in subsequent analyses.

181

182 *Using wzi-Seq to Investigate Klebsiella Colonization Dynamics*

183 To determine the frequency of mono- or mixed colonization, we analyzed a previously
184 constructed case-control cohort of *Klebsiella*-colonized patients, where cases developed an
185 extraintestinal infection and controls did not[13,20]. A total of 236 rectal swabs were subjected to
186 *wzi*-Seq to determine the frequency of mono- or mixed colonization. Strict criteria for strain
187 detection were applied, including that the abundance must be >1% and that *wzi*-Seq results had
188 to detect *wzi* types previously found by culture and Sanger sequencing of individual isolates
189 from the same rectal swab (see methods). Out of 236 rectal swabs, 27/236 (11.4%) failed *wzi*
190 PCR, 28/236 (11.9%) failed quality control (see methods), 120/236 (50.8%) samples required a
191 second round of *wzi* PCR to meet the DNA input requirements for ONT sequencing, and 10/236

192 (4.2%) were considered inconclusive. A total of 171 (72.5%) samples passed all quality control
193 metrics and were included in the final analysis. Of these 171 samples, 108/171 (63.2%) were
194 mono-colonized with only one *wzi* type detected and 63/171 (36.8%) were considered mixed
195 with 2-7 *wzi* types present. This contrasts with only 5/171 (2.9%) samples detected as mixed by
196 conventional culture and *wzi* typing of 3 isolates per rectal swab. The majority of mixed samples
197 (79.4%) were colonized with 2 or 3 unique *wzi* types (Fig. 4). Across 171 mono- and mixed
198 colonized samples, 160 unique *wzi* types were identified.

199

200 To further investigate the colonization dynamics in patients colonized with multiple *wzi* types, the
201 abundances of *wzi* types were investigated in mixed samples (Fig. 5). The most abundant of the
202 *wzi* types present (*wzi* Rank 1) had an abundance greater than 50% in 58/63 (92.1%) and
203 greater than 75% in 42/63 (66.7%) of samples (Fig. 6A), indicating that even in mixed samples
204 one *wzi* type tended to dominate (Fig. 6A). Interestingly, there was high diversity in the *wzi* types
205 detected in mixed samples, with 119 unique *wzi* types detected (Fig. 6B,C). There was not a
206 single *wzi* type that tended to be the dominant colonizer, as there were 50 unique *wzi* types
207 detected across mixed samples as the highest *wzi* type (*wzi* Rank 1), and dominant strains in
208 one sample were subdominant in another (e.g. *wzi* 84 in pink, Fig. 6C).

209

210 *Validation of Rectal Swab wzi-Seq Results*

211 To confirm the detection of mixed colonization, repeatability and agreement with an orthogonal
212 method were assessed. The *wzi*-Seq analysis was repeated on a separate sample aliquot and
213 compared to direct Illumina sequencing from the same aliquot. Eight swab samples, four mixed
214 and four mono-colonized based on analysis above, were struck out on MacConkey agar to
215 select for Gram-negative bacteria including *Klebsiella* and then DNA was extracted from plate

216 sweeps for parallel analysis. The repeat *wzi*-Seq analysis reproduced the categorization of
217 mixed and mono-colonization, excluding one sample that failed sequencing (Suppl. Table 5).
218 Data from direct Illumina sequencing of plate sweeps detected *Klebsiella* species in each
219 sample except for 2 samples (Suppl. Fig. 1). For samples with *Klebsiella* detected, reads were
220 mapped to the *wzi-1* reference sequence and *wzi* types were inferred to be present in the
221 population using a popANI approach (see methods). Overall, the results were highly consistent
222 with the rectal swab and plate sweep, with all error0.005 *wzi* types detected, and the same
223 categorization of mixed and mono-colonization (Suppl. Table 5).

224

225 Some *Escherichia coli* (*E. coli)* strains can have a *Klebsiella* like capsule and be amplified with
226 *wzi* primers[21,22]. To determine whether *wzi*-Seq was impacted by non-specific amplification of *E.*
227 *coli wzi* we performed additional bioinformatic analysis. We first evaluated the nucleotide
228 similarity of all 741 unique *Klebsiella wzi* types against all known *E. coli* genomes in the NCBI
229 database using BLAST. Out of all 741 *wzi* types, 6 *wzi* types had 100% identity to known *E. coli*
230 genomes. *E.coli* DNA sequences that had significant but <100% match with any of the 741
231 *Klebsiella* reference *wzi* types were added to our existing *wzi* reference database. Then the *wzi*-
232 Seq analysis pipeline using the updated reference database was rerun on rectal swabs with
233 mixed colonization (see methods). Out of 63 mixed samples, 62/63 (98.4%) were still classified
234 as mixed after running the *E. coli* amended pipeline; one had a second *Klebsiella*-specific *wzi*
235 type that fell to 0.9%, just below our 1% cutoff for a mixed sample. Five samples contained at
236 least one *wzi* type (wzi_5, wzi_370, or wzi_173) that was 100% identical to an *E. coli wzi* type,
237 but this did not change the result of mixed detection because either this *wzi* type was from a
238 culture-confirmed *K. pneumoniae* isolate or there were multiple *Klebsiella*-specific *wzi* types
239 present in the sample (Supplemental Dataset).

240

241 *Mono-colonization is Associated with an Increased Risk of Klebsiella Infection*

242 To determine whether mono- or mixed colonization was associated with an increased risk of
243 developing a *Klebsiella* infection, patient characteristics associated with each rectal swab were
244 evaluated. Out of 171 rectal swabs, 66 were from colonized patients who developed a
245 subsequent *Klebsiella* infection (case) while 105 were from asymptomatic colonized controls
246 (Suppl. Fig. 2A). Mixed colonization was more prevalent in control samples (45/105, 43%) than
247 in cases (18/66, 27%) (Suppl. Fig. 2A, B).

248

249 *Klebsiella* dominance in the gut is associated with an increased risk of infection, so we
250 compared *Klebsiella* abundance in mono- and mixed colonization as previously measured by
251 our validated qPCR assay[7,8,11]. *Klebsiella* relative abundance in mono-colonized samples was
252 significantly higher in cases than controls but this was not the case in mixed colonized samples
253 (Suppl. Fig. 3A, B). Overall, the relative abundance was significantly higher in mono- than in
254 mixed colonized patients (Suppl. Fig. 3C).

255

256 To determine the association of mono- and mixed colonization with subsequent infection, we
257 constructed a multivariable model. Patient characteristics could be putative confounders to
258 consider for adjustment (see methods), so we characterized the distribution of these variables
259 versus subsequent infection. Table 2 shows important baseline characteristics of the study
260 population and variables that were considered in our final model. Relative abundance and
261 mono/mixed colonization status were found to be colinear. If they lie along the same causal
262 pathway and with the observed collinearity, then including *both* relative abundance and mono-
263 colonization in the model together would result in weakening the association. Indeed, we
264 observed that while both mono-colonization and relative abundance are both associated with

265 infection (Table 2), including both as covariates weakens the association with infection and there
266 is no interaction noted statistically (p=0.691 for the interaction vs. infection in a multiple logit
267 model). Therefore, relative abundance was not considered for the final model, and we focused
268 on mono-colonization vs. mixed colonization[11,13]. Other than age, gender and collection month,
269 which were *a priori* factors to include for adjustment (see methods), only prior urinary catheter
270 use and baseline serum albumin were retained in the final model. After adjustment for these
271 potential confounders (Suppl. Table 6) we measured an independent association between
272 mono-colonization and subsequent infection demonstrating a 2.18-fold increased odds of
273 infection (95% CI 1.08-4.57; p=0.034).

274

275 *Gut Community Structures Do Not Vary Between Mono or Mixed Colonized Patients*

276 To investigate whether overall microbial community differences contribute to *Klebsiella*
277 colonization dynamics, we analyzed available 16S rRNA gene sequencing of the patient rectal
278 swabs[23]. Interestingly, the gut microbial community structure was not significantly different
279 between mono- or mixed colonized patients. No significant differences in community richness,
280 evenness, or diversity were observed between patient groups (Fig. 7A-C). Furthermore, a
281 principal coordinate analysis did not reveal any significant differences in the beta-diversity
282 between mono- and mixed colonized patients (Fig. 7D). When using 16S rRNA data to assess
283 *Klebsiella* abundance, higher abundance of the ASV: ASV000001 *Klebsiella* was observed in
284 mono compared to mixed samples that approached statistical significance (p=0.06; Fig. 7E),
285 consistent with our qPCR data.

286

287 **Discussion**

288 In this study, we describe the development and validation of *wzi*-Seq, a novel amplicon-based
289 sequencing method to measure *Kp* population structure in samples (Fig. 1). We demonstrate
290 that the *wzi*-Seq method has excellent read accuracy and high precision for measuring relative
291 abundance, and these performance characteristics are not impacted when performing additional
292 rounds of PCR (Fig. 2; Table 1; Suppl. Tables 1-3). Furthermore, the assay has a sensitivity and
293 specificity of 93/99.8% for the error0.005 pipeline and 94.21/100% for the error0 pipeline for
294 strain detection in samples with up to 58 strains (Fig. 3; Suppl. Table 4). Applying *wzi*-Seq to a
295 cohort of *Kp* colonized patients, we determined that *Kp* mono-colonization was more prevalent
296 than mixed colonization, with mono-colonization being independently associated with an
297 increased infection risk (OR=2.18; p=0.034). This study describes a novel and reliable
298 technique to investigate *Kp* colonization dynamics and reveals the population structure of
299 colonizing strains as a potentially important risk factor for infection.

300

301 The *wzi*-Seq assay has performance characteristics that make it suitable for several
302 applications. This amplicon sequencing approach can perform strain typing directly from
303 samples, distinguishing it from WGS, MLST, PGEF, and MALDI-TOF that typically require
304 culturing of single *Klebsiella* isolates. Importantly, *wzi* typing has similar discriminatory power as
305 MLST and K-typing[7,16,24]. Also, the assay can accurately detect and quantify mixtures of *Kp*
306 strains within a sample, similarly to 16S rRNA gene sequencing for multiple species. We
307 developed the *wzi*-Seq assay using nanopore sequencing in part because the long region of
308 interest precluded Illumina sequencing. We have taken advantage of recent advances in
309 nanopore accuracy at both the sequencing and base calling steps, and mitigated the residual
310 error rate by using read mapping to known sequences and applying several quality filtering
311 steps[25]. As used here, this method can be used to detect and quantify multiple strains in a
312 sample with high confidence.

313 The finding that mixed colonization occurs in a substantial subset of patients, but that mono-
314 colonization is associated with increased infection risk has several implications. Mixed
315 colonization and infection has been documented in other pathogens including *Clostridioides*
316 *difficile*[26–29]. Unlike *Klebsiella,* C. *difficile* causes intestinal disease and mixed infection with
317 multiple strains was significantly associated (OR 3.5, 95% CI 1.3-9.4, p = 0.015) with recurrence
318 in patients, even after adjustment for gender and previous exposure to antibiotics[26]. For
319 *Klebsiella*, mixed colonization may represent a more commensal state than mono-colonization.
320 Indeed, a recent study illustrated that two strains of *Kp* can cross-feed one another with one
321 strain compensating for the fact that the other cannot utilize a specific nutrient[30,31]. This may
322 represent a stable colonization state where one strain is kept in check by its reliance on the
323 other, reducing the chance that they cause clinical disease. In contrast, mono-colonization was
324 associated with higher relative abundance of *Kp*, and these variables were co-linear in our
325 models of infection. This may represent the outcome of competition with other *Kp* strains,
326 resulting in complete displacement of one strain and replacement with the other. Even in mixed
327 colonization, one *wzi* type tended to dominate, suggesting that *Kp* strains outcompete other
328 strains, or at least settle at a higher density in equilibrium. It would be interesting to explore
329 whether mixed colonized patients are at higher risk for prolonged colonization, or whether over
330 time these patients progress to a mono-colonized state due to dominance by a single strain.
331 Furthermore, this highlights that typing only single isolates may miss these mixed colonized
332 states with implications not only on patient infection risk but also potential transmission events.

333

334 We also found that the gut microbial community was not significantly different between mono-
335 and mixed colonized patients, suggesting that microbial richness or diversity in the gut does not
336 impact strain colonization status (Fig. 7). This is consistent with previous studies that only
337 observed minor changes in the gut microbial community during *Kp* colonization in mice[32–34]. This

338 suggests that in our patient cohort, other clinical and environmental factors might play a more
339 significant role in shaping the overall community structure. Alternatively, *Kp* strains might occupy
340 a particular ecological niche within the gut microbiome that allows coexistence with other
341 microbes without causing substantial shifts in the community structure.

342

343 This study and the *wzi*-Seq method have several limitations. As *wzi*-Seq relies on a single
344 conserved gene for discrimination, the technique cannot be used to differentiate between strains
345 of the same *wzi* type. Furthermore, the primers used to amplify the *wzi* gene can, in rare cases,
346 amplify group 1 *E. coli wzi* genes[21,22]. The incidence of *Klebsiella* capsules in *E. coli* is unclear,
347 although a study of freshwater *E. coli* found *Klebsiella* capsules in only 7% of isolates[35]. We
348 addressed this limitation by including *E. coli wzi* sequences in our reference database for read-
349 mapping and requiring a 100% match to a *Klebsiella wzi* sequence to consider that *wzi* type to
350 be present in a given sample. When using complex samples with unknown *wzi* types,
351 orthogonal validation approaches may be needed to distinguish *Kp* and *E. coli* with identical *wzi*
352 sequences. In conclusion, this study describes the development and validation of *wzi*-Seq, a
353 novel technique that enables the detection and quantification of *Kp* from complex samples. In
354 future directions, the *wzi*-Seq technique can be applied to test patient samples, adapted to
355 target additional genes, and applied to characterize diverse populations of *Kp* in both
356 experimental and natural conditions.

357

358 **Methods**

359 **Rectal Swab Sample Collection and Analysis**

360 Patient enrollment and sample collection was performed previously and was approved by the
361 Institutional Review Board[13,20]. Patient rectal swabs were collected with instructions to pass the

362 anal verge using flocked swabs placed in Amies transport media (Becton, Dickinson, Franklin
363 Lakes, NJ), and screened for *Klebsiella* via plating on MacConkey agar and verification via
364 MALDI TOF mass spectrometry[13]. From positive rectal swabs, up to three *Klebsiella* isolates
365 were collected and subject to *wzi* PCR and Sanger sequencing and the *wzi* type of each isolate
366 was determined by matching the consensus *wzi* sequence to the BigsDB database
367 (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html).

368 ***wzi*-Seq Assay**

369 DNA was extracted from rectal swabs or contrived samples using the Qiagen UltraClean
370 Microbial DNA kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. PCR was
371 performed using previously published *wzi* primers and the Q5 polymerase (New England
372 Biolabs, Ipswich, MA, USA) with volumes as specified by the manufacturer in a 100 µL
373 reaction[16]. For all reactions, 4 µL of DNA template with a concentration of 1-2ng/µL was used.
374 PCR conditions were 30 sec at 98°C, then 30 cycles of 10 sec at 98°C, 30 sec at 59°C, 30 sec
375 at 72°C, followed by 2 minutes at 72°C on a MiniAmp Plus Thermal Cycler (Thermo Fisher
376 Scientific, Waltham, MA, USA). For samples with poor amplification a second round of PCR was
377 performed using the same conditions as listed above, using 4µL of the first PCR reaction as the
378 template. Samples were run on a 1% agarose gel at 100V for 30 minutes to confirm
379 amplification, purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), and
380 quantified using Nanodrop and Qubit (Thermo Fisher Scientific, Waltham, MA, USA). All
381 samples were submitted to the University of Michigan Advanced Genomics Core (AGC) for
382 Oxford Nanopore Technologies sequencing (Oxford Nanopore Technology, Oxford, UK).
383 Basecalling was performed using the SUP pipeline by the University of Michigan AGC.

384 **Bioinformatic analysis**

385 *error0 and error0.005 Pipelines*

386 Reads with mean base quality lower than 10, or with more than 5% bases lower than 10 were
387 removed using NanoFilt v2.8.0[36] and a custom script. Remaining reads were aligned against the
388 reference *wzi* types curated from the BIGSdb-Pasteur database
389 (https://bigsdb.pasteur.fr/klebsiella/) using Minimap2 v2.26[37]. Reads were filtered by these
390 criteria sequentially: mapping quality was larger than zero; the read had a single mapping
391 location after mapping quality filtering; alignment covered more than 95% of the mapped gene
392 sequence; NM value (number of mismatches and gaps) was zero. Filtered reads were counted
393 on each reference gene sequence.

394 The filtering for the error0.005 pipeline was the same as the error0 pipeline except that the base
395 quality fraction filter was disabled, and the error rate filter was relaxed to lower than 0.5% of the
396 alignment length.

397 *Quality criteria for rectal swab samples*

398 To ensure confidence in identified *wzi* types the following quality control metrics were applied to
399 all samples: 1) error0.005 samples had to have greater than 1,000 reads, 2) error0.005 and
400 error0 readfrac cutoffs (>0.00275 and >0.00175, respectively) were applied before abundance
401 calculations, 3) samples had to detect *wzi* types from banked rectal isolates from that sample
402 (168/171 available), and 4) *wzi* type abundance had to be greater than 1%. A 1% abundance
403 cutoff was applied as this was the lowest abundance validated using contrived mixtures. To
404 reduce the possibility of contamination causing the appearance of mixed colonization, samples
405 were considered inconclusive and not included if the wzi_2 allele of our commonly used
406 laboratory strain was present as the first or second most abundant *wzi* type and was not
407 detected via rectal isolate typing. Samples were then examined for concordance between
408 error0.005 and error0 pipelines. If there was discordance, BAM files were examined in IGV for
409 potential single nucleotide polymorphisms that may explain the discordance and where
410 possible, checked against sequenced genomes from that specimen.

411 *Detection of potential E. coli capsule sequences*

412 To map reads to a reference database with both *Klebsiella* and potential *E. coli wzi* alleles, the
413 error0 pipeline was run with the following changes: 1) the mapping quality filter was removed, 2)
414 zero mismatches were allowed between mapped reads and the reference, 3) 100% coverage of
415 the reference was required, and 4) a unique mapping position needed to remain after steps 2
416 and 3. Mixed samples were re-run using this *E. coli* amended pipeline to determine whether this
417 impacted the mixed status of any samples.

418

419 **Illumina Sequencing Analysis**

420 *Data QC and determination of species composition*

421 Fastqc (v0.12.1) (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to
422 assess the quality of sequencing reads before and after trimming with Trimmomatic (v0.36)[38].
423 InStrain (v1.10.0) was used to identify bacterial species composition for each population
424 sample[39]. The reference database consisted of 4,644 representative genomes from the Unified
425 Human Gastrointestinal Genome (UHGG) collection in 2025. Trimmed reads were aligned to
426 each reference independently with Bowtie2 (v2.4.2). inStrain output was filtered with 50%
427 genome breadth cutoff.

428 *wzi popANI pipeline*

429 BWA mem (v0.7.17) (https://arxiv.org/abs/1303.3997), was used to align trimmed reads to *wzi-1*.
430 Samtools (v 1.21) was used to transform sam files into bam files and index the bam files[40]. R
431 (v4.4.3) was used to calculate the popANI between population sequences and wzi-references,
432 which provides the lower-bound distance to each *wzi* type. The value of popANI to a given wzi-
433 type increases if there are no shared alleles between a sample and *wzi* reference at a given

434 position in the alignment. For each sample, *wzi* types were removed if popANI was >3 or higher
435 than the lowest popANI value. Breadth of coverage was estimated for each remaining *wzi* type
436 and *wzi* types were removed if breadth of coverage was lower than 99%.

437

438 **16S rRNA sequencing and analysis**

439 DNA extraction and 16S rRNA gene sequencing was performed elsewhere[23]. Sequences were
440 processed with mothur (v. 1.48.0) and aligned to the SILVA reference alignment, release
441 132[41,42]. The sequencing error rate was assessed using a predefined mock community and
442 estimated to be 0.0066%. For all analyses, sample read counts were rarefied to 8,363 reads
443 (lowest sample read count >8,000), and one sample (PR24765, prokka_sample_923-JV-132,
444 rectal swab corresponding to patient colonized by *Klebsiella* strain Kp7994) was excluded due
445 to low total read count (<8,000). Differences in community structure were assessed by
446 PERMANOVA (1,000 permutations) using robust Aitchison distance as the dissimilarity metric
447 from the vegan package, v. 2.6-2, and alpha diversity metrics were calculated using mothur[41,43].
448 Data analysis was carried out in RStudio 2021.09.0+351 "Ghost Orchid" Release for macOS.
449 For all analyses, a p-value ≤ 0.05, after Benjamini-Hochberg adjustment where appropriate, was
450 considered statistically significant. All scripts for 16S rRNA gene sequencing analysis used for
451 this study are available at https://github.com/jayvorn/wzi_mixed_mono_colonization (embargoed
452 until publication).

453 Logistic Regression Model and Analysis

454 The goal of this analysis was to determine the independent contribution of mono-colonization
455 vs. mixed colonization with *Kp* to risk of subsequent clinical infection from *Kp*. We did this by
456 including mono-colonization as the primary predictor and then included covariates due to a priori
457 importance (e.g., past matching criteria) or based on retention as putative confounders after a

458 stepwise selection procedure. For the former types of covariates, our prior studies on the same
459 cohort from which the current study's subjects were drawn, informed the clinical variables to
460 consider for adjustment[20]. Notably, since our source cohort was from a matched case control
461 study but not all cases and controls could be included here, we included the matching criteria of
462 age, gender, and month of swab collection as covariates for adjustment in our model[13]. We then
463 turned to purposeful selection to determine which other putative confounders to include in the
464 final model[44]. Starting with variables capturing demographics, comorbidities, prior/baseline
465 medication and device exposures, and prior laboratory parameters, we proceeded in a stepwise
466 fashion until only significant variables and potential confounders were retained, and this is the
467 final model presented in the results[44].

468 **Data availability**

469 The 16S sequencing data used in this study are deposited in the Sequence Read Archive (SRA)
470 database under accession code PRJNA789565. The *wzi*-sequencing data will be deposited and
471 available in SRA prior to publication. Patient variables used in logistic regression can be made
472 available upon request, after ensuring de-identification and compliance with IRB regulations at
473 both institutions.

474 **Funding Statement**

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476 Infectious Diseases of the National Institutes of Health under Award Number R21AI176642. The
477 content is solely the responsibility of the authors and does not necessarily represent the official
478 views of the National Institutes of Health.

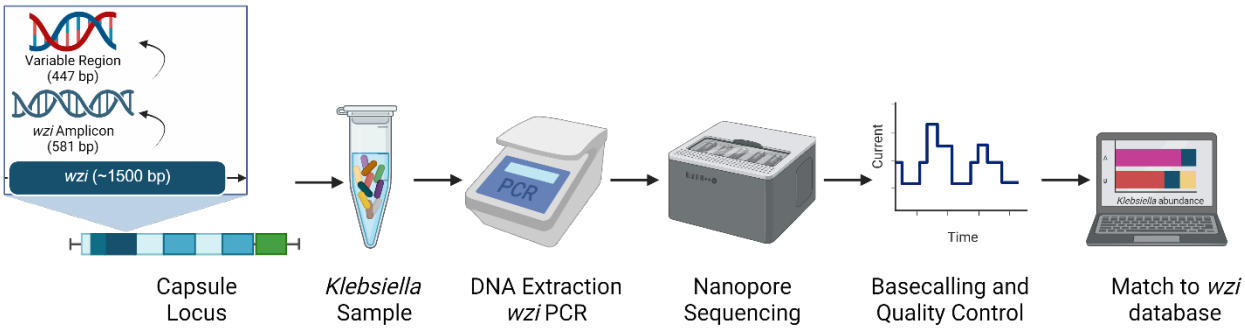
479

480

481 **Competing Interests**

482 Dr. Vornhagen has consulted for Vedanta Biosciences, Inc. Dr. Rao has received an
483 investigator-initiated grant from Merck & Co, Inc.; he has consulted for Seres Therapeutics, Inc.,
484 Rebiotix, Inc., Vedanta Biosciences, Inc., and Summit Therapeutics, Inc.

485 **Figures**



486

487 Figure 1: Overview of the *wzi*-Seq method

488 A 581 bp *wzi* amplicon generated using previously published *wzi* primers, and the 447 bp
489 variable region of the *wzi* amplicon are used to distinguish different *wzi* types. The *wzi*-Seq
490 method starts with a *Klebsiella* sample with known or unknown *wzi* types which is then subject
491 to DNA extraction and *wzi* PCR. Nanopore sequencing of the *wzi* amplicon is performed,
492 followed by base calling and quality control of reads to ensure read accuracy. All reads that pass
493 quality control and filtering are then aligned against a database of 741 known *wzi* type reference
494 sequences to calculate the abundance of each *Klebsiella wzi* type in the sample.

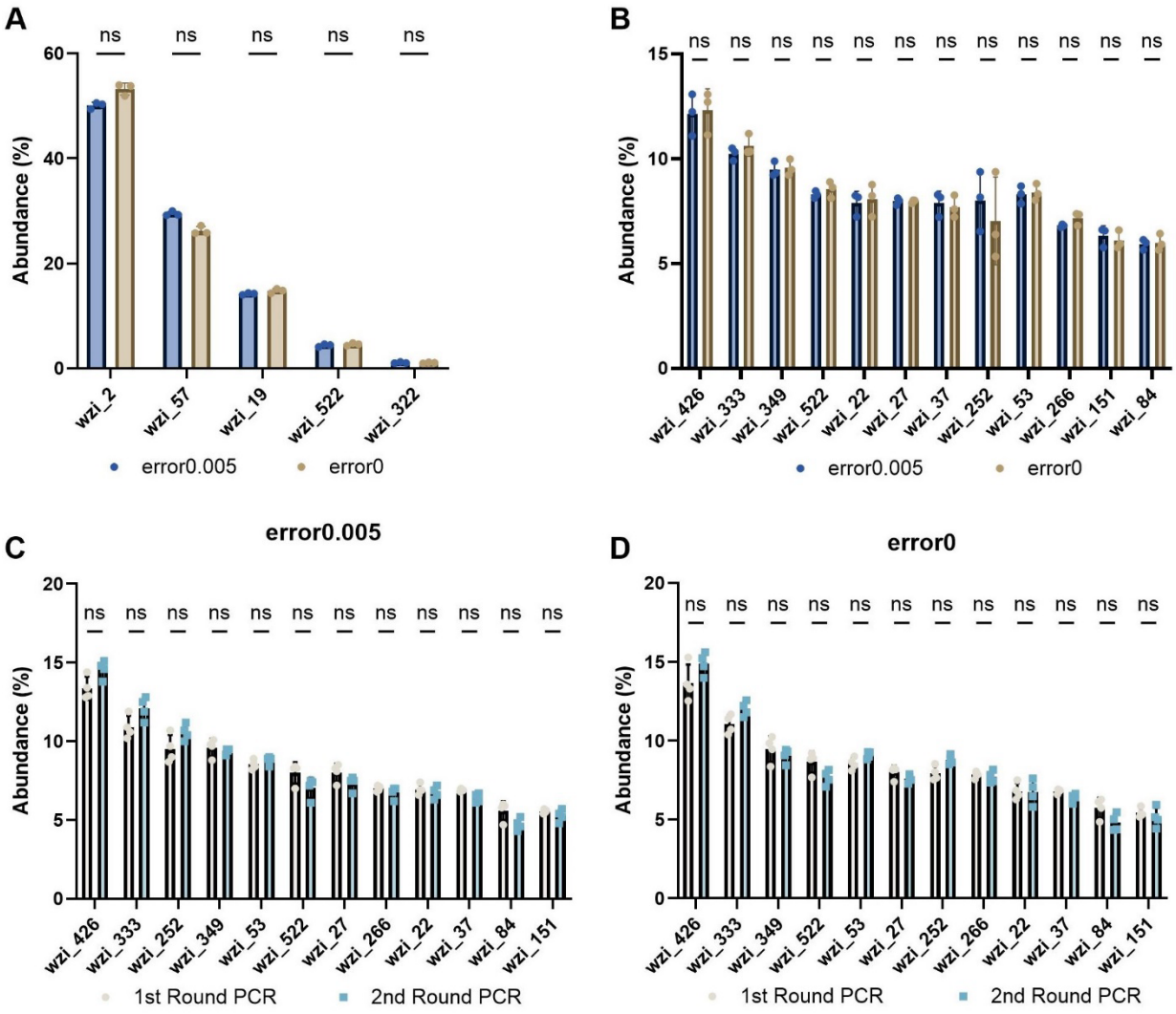
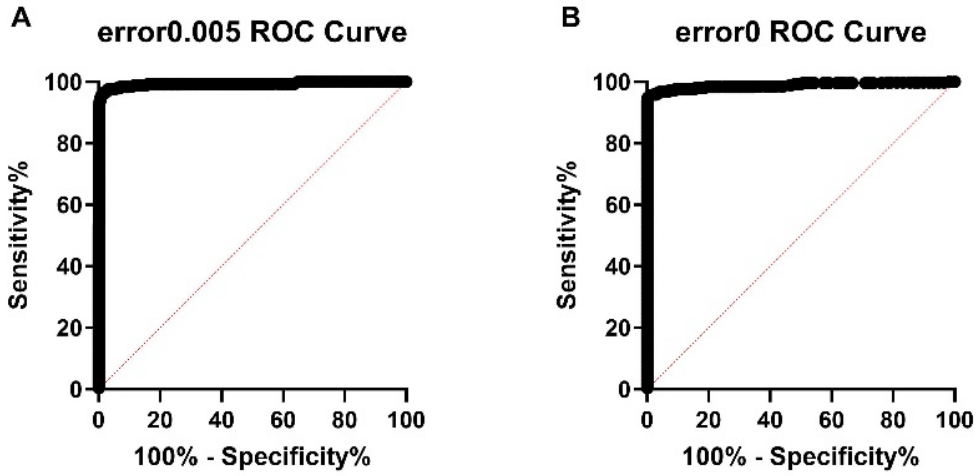


Figure 2: Abundance values are equivalent across pipelines and rounds of PCR

(A) *wzi*-Seq was performed on a mixture of 5 *wzi* types wzi_2, wzi_57, wzi_19, wzi_522, and wzi_322 with expected relative abundances of 50, 30, 15, 5, and 1%, respectively. The mean abundance of each *wzi* type was determined from three replicates using the error0 and error0.005 pipelines. The abundance of each *wzi* type was compared between the two pipelines and there were no significant differences in the abundance for each *wzi* type as determined by the error0.005 or error0 pipelines. (B) *wzi*-Seq was performed on a mixture of 12 unique *wzi* types and the mean abundance of each *wzi* type was quantified by either the error0 or error0.005 pipeline. (C) A 12 *wzi* type mixture was subject to either one or two rounds of *wzi* PCR, with each round consisting of 40 cycles. The 12 *wzi* type mixtures subject to one or two rounds of PCR were analyzed using both the error0 (C) and error0.005 (D) pipelines to calculate the abundance of each *wzi* type. For (A-D), a multiple Wilcoxon paired t-test was used with an n≥3.



C

	True Positives	False Positives	ROC Area	Cutoff	Sensitivity/ Specificity (%)	Likelihood Ratio
error0.005	486	1492	0.9925	> 0.002752	93/99.8	462.5
error0	484	810	0.9886	>0.001722	94.21/100	NA

Figure 3: The *wzi*-Seq technique is highly sensitive and specific for detection of strains in mixed samples

(A-B) A total of 23 contrived samples containing between 5-58 unique *wzi* types were used as input for the receiver operating characteristic (ROC) analysis using abundance data (readfrac) from both the error0 and error0.005 pipelines. (C) The total number of true positive and false positive values used to generate the ROC curve and ROC curve area is displayed. Using the ROC analysis, readfrac cutoffs with maximum specificity while retaining high sensitivity were chosen for both the error0.005 and error0 pipelines.

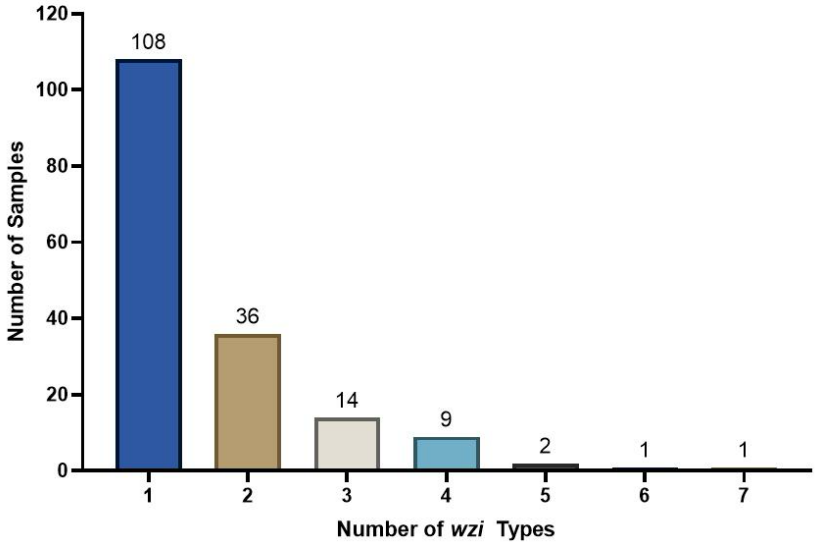


Figure 4: Mono-colonization is more prevalent than mixed colonization in patient rectal swabs

The *wzi*-Seq method was used to detect the number of unique *wzi* types present in *Klebsiella* colonized patients from a previously published nested case-control study. Mono-colonization was more prevalent (63.2%) than mixed colonization (36.8%) with mixed samples having been 2-7 unique different *wzi* types.

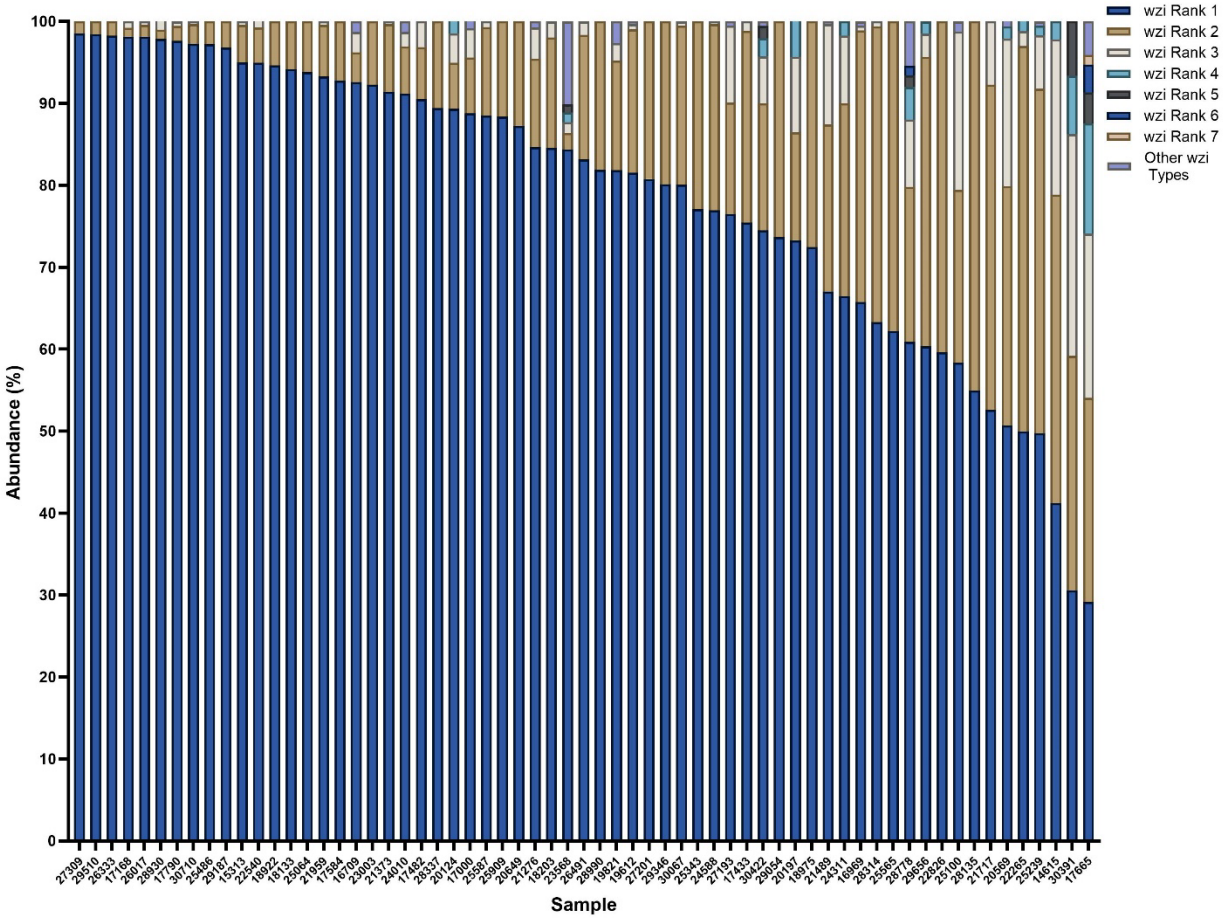


Figure 5: Mixed colonized patient samples are commonly dominated by one *wzi* type

The abundances of *wzi* types identified in all mixed samples were quantified with *wzi* rank 1 being the *wzi* type highest in abundance in each sample and *wzi* rank 7 being the lowest in each sample. All *wzi* types with an abundance less than 1% were labeled as “other”.

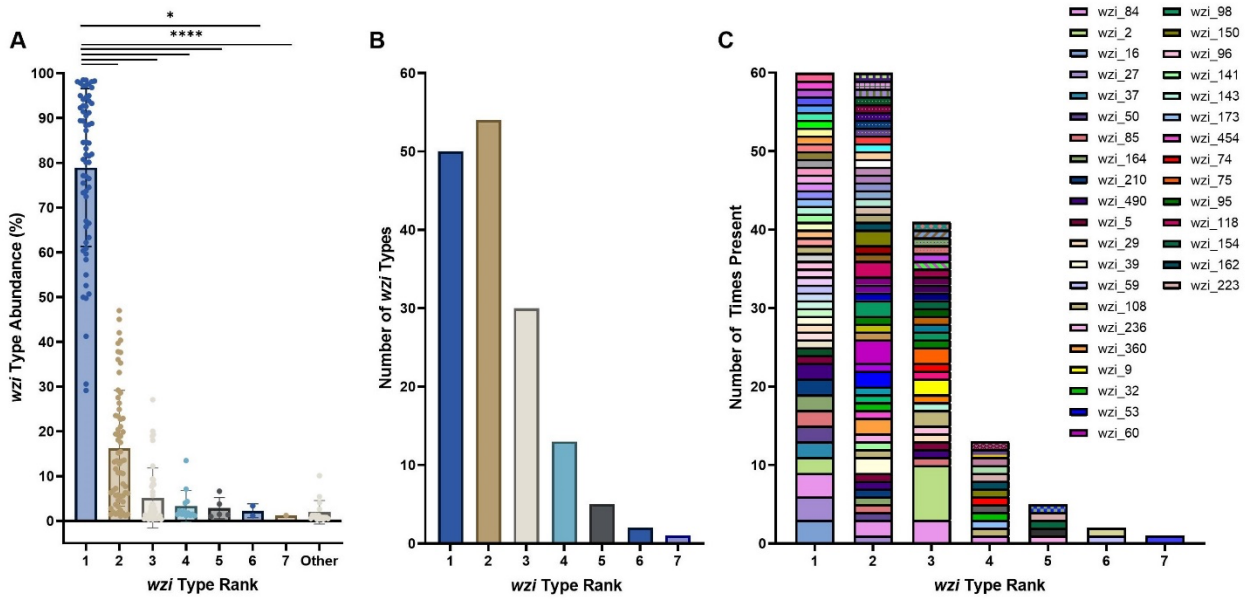


Figure 6: Mixed colonized patients are colonized by diverse *wzi* types

The abundance of each *wzi* type based on the abundance ranking was determined. Across all samples, the *wzi* types in rank 1 had significantly higher abundances than any of the other detected *wzi* types, highlighting that mixed samples were uneven with a single *wzi* type dominating samples (A). Across all samples the number of unique *wzi* types present based on the abundance ranking was determined (B). The number of times each unique *wzi* type was present across all samples was quantified in order of the *wzi* type highest to lowest in abundance across all samples (C). The legend displays only colors for *wzi* types that were present more than once in each category. For (A), *, $p < 0.05$; ****, $p < 0.0001$, by Kruskal-Wallis t-test and n=63.

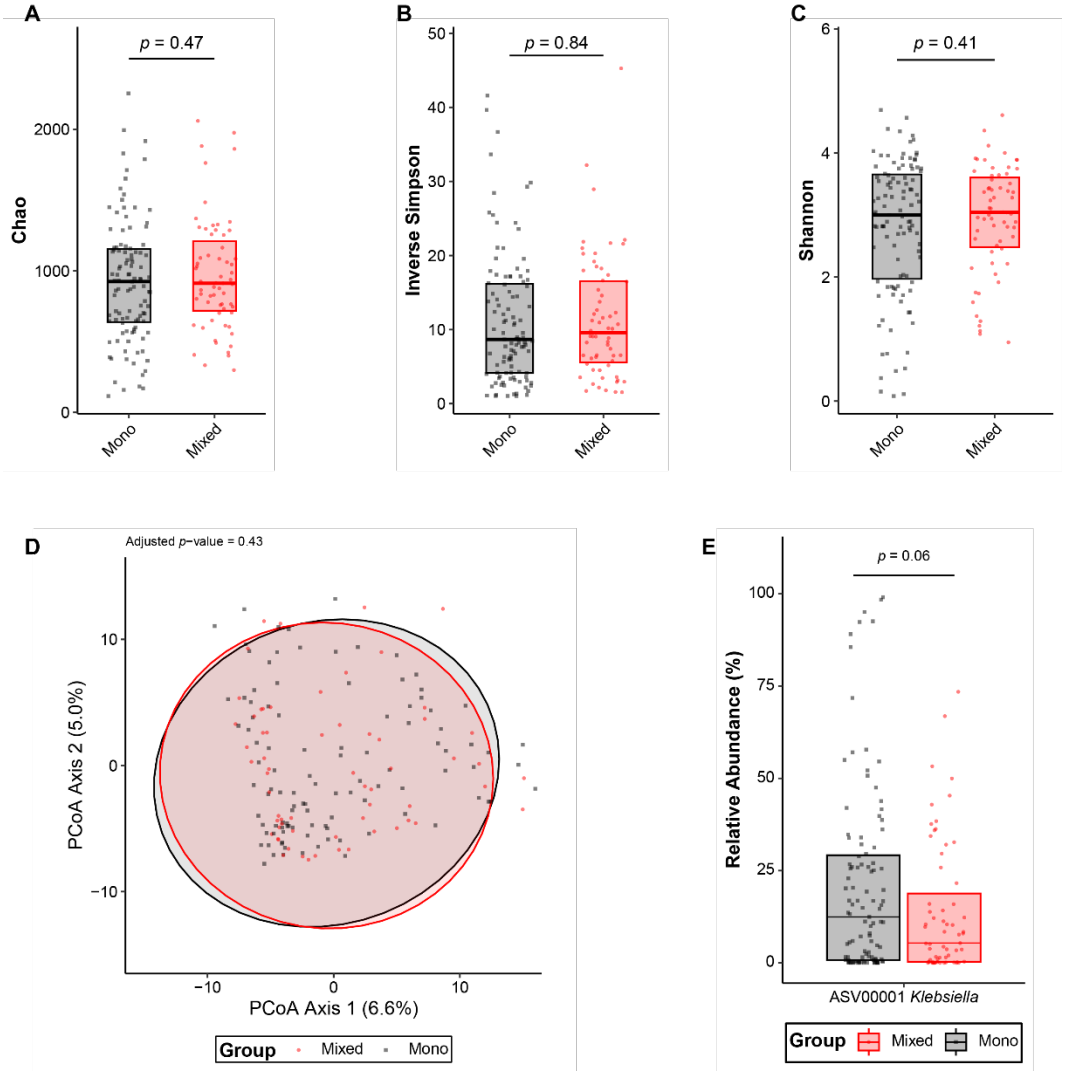


Figure 7: Gut community structures do not differ significantly between mono- or mixed colonized patients

The gut community structure using alpha (A-C) and beta (D) diversity metrics were assessed via 16S sequencing of patient rectal swabs and the bioinformatic package, mothur. No significant differences were in alpha diversity metrics including Chao (A), inverse Simpson (B), or Shannon were observed (C). There were no significant differences in beta diversity (D) between mono- or mixed colonized patients. Based on ASVs higher relative abundance levels (E) were observed in mono- compared to mixed colonized patients but this did not reach statistical significance. For all analyses, a p-value ≤ 0.05, after Benjamini-Hochberg adjustment where appropriate was considered significant.

558 **Tables**

559 Table 1: Summary table of expected and experimental abundance values for 5 *wzi* type samples
560 based on pipeline and rounds of PCR

Pipeline		error0.005		error0		error0.005			
PCR		1 Round		1 Round		1 Round		2 Rounds	
wzi Type	Expected Abundance (%)	Mean Abundance (%)	Standard Deviation	Mean Abundance (%)	Standard Deviation	Mean Abundance (%)	Standard Deviation	Mean Abundance (%)	Standard Deviation
2	50	50.1	0.6	53.2	1.1	48.8	0.36	45.9	0.20
57	30	29.5	0.4	26.3	0.8	32.1	0.41	35.2	0.17
19	15	14.2	0.2	14.8	0.4	13.7	0.16	11.5	0.04
522	4	4.4	0.3	4.6	0.2	4.0	0.04	4.0	0.05
322	1	1.1	0.1	1.1	0.1	1.1	0.14	1.9	0.05

561

562

563 Table 2: Case-control study population characteristics

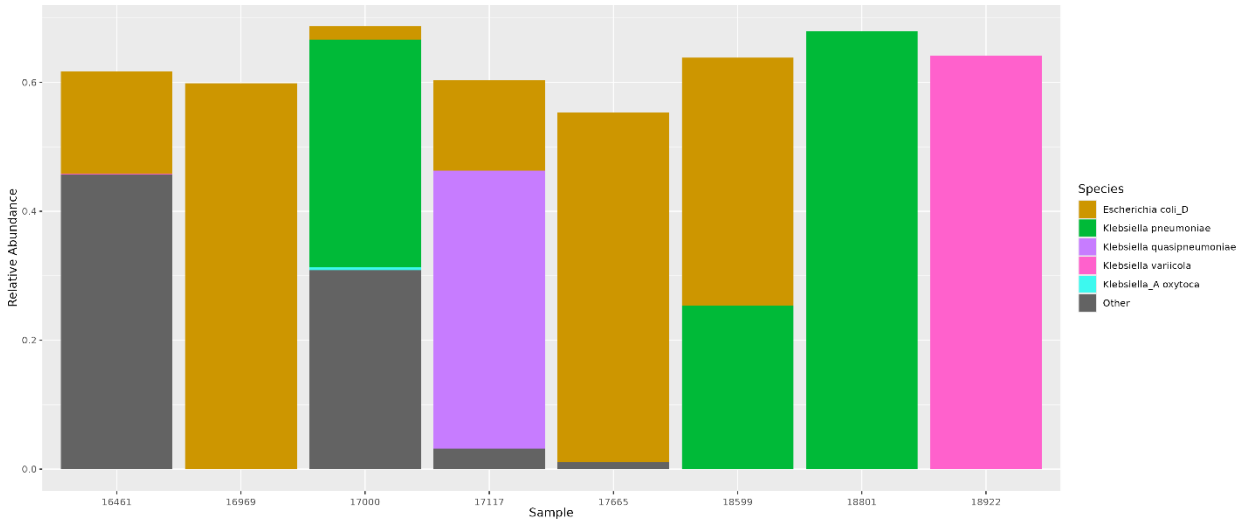
Characteristic	No infection[1] N = 105	Infection[1] N = 66	p-value[2]
Age[3]	61 (54, 70)	59 (52, 69)	0.6
Gender[3]			>0.9
Female	49 (47%)	31 (47%)	
Male	56 (53%)	35 (53%)	
Race			0.8
Non-white	21 (20%)	12 (18%)	
White	84 (80%)	54 (82%)	
Colonization[3]			0.04
Mixed	45 (43%)	18 (27%)	
Mono	60 (57%)	48 (73%)	
***Klebsiella* relative abundance**	4 (0, 20)	20 (1, 59)	0.002
Weighted Elixhauser Score	19 (12, 26)	23 (16, 28)	0.06
Depression	25 (24%)	25 (38%)	0.049
Prior/Baseline Urinary Catheter[3]			0.006
FALSE	49 (47%)	17 (26%)	
TRUE	56 (53%)	49 (74%)	
Prior/Baseline Feeding Tube			0.056
FALSE	103 (98%)	60 (91%)	
TRUE	2 (1.9%)	6 (9.1%)	
Prior Broad Spectrum Antibiotic Exposure	19 (18%)	22 (33%)	0.023
Prior/Baseline Pressor Dependent Hypotension	11 (10%)	15 (23%)	0.03
Baseline Serum Albumin[3]			0.003
<2.5	21 (20%)	29 (44%)	
≥2.5	79 (75%)	34 (52%)	
NA	5 (4.8%)	3 (4.5%)	

[1]Results reported as median (Q1, Q3) or n (%) as appropriate

[2]Wilcoxon rank sum test, Pearson's Chi-squared test, or Fisher's exact test as appropriate

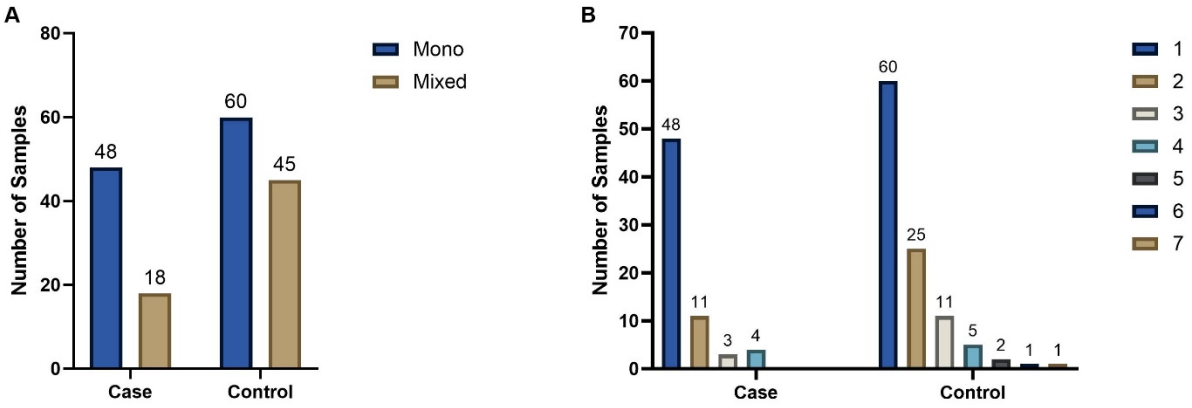
564 [3]These variables were included in the final multivariable model.

Supplemental Figures



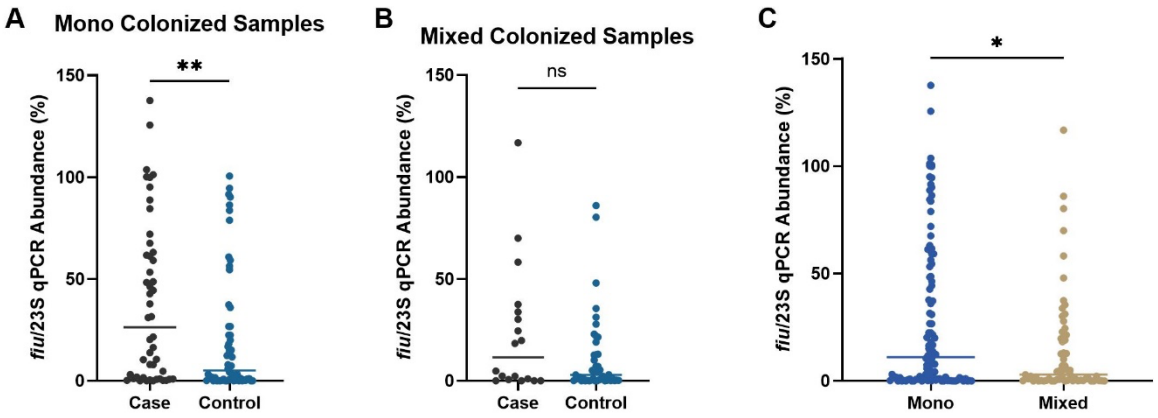
Supplemental Figure 1: Species composition based on Illumina Sequencing of *Klebsiella* Mixed and Mono-Colonized Rectal Swabs used for validation.

Eight glycerol amended swab samples, four mixed and four mono-colonized based on analysis above, were struck out on MacConkey agar to select for Gram-negative and then DNA was extracted. Samples were subjected to Illumina sequencing and bacterial species composition was determined by InStrain.



Supplemental Figure 2: Mono- and mixed colonization in cases of *Klebsiella* infection and controls

Colonization and case status of all patients were evaluated based on mono- or mixed colonization status (A) or the number of *wzi* types present in each sample (B).



Supplemental Figure 3: Mono-colonized case samples have significantly higher *Klebsiella* relative abundance than controls

(A-C) The *Klebsiella* relative abundance in each rectal swab was quantified using a validated qPCR assay quantifying the conserved *Klebsiella fiu* gene and the panbacterial 23S rRNA gene. The ratio of the *fiu* and 23S rRNA gene were used to calculate the *Klebsiella* relative abundance in case or control patient rectal swabs from mono- (A) or mixed (B) colonized patients. The *Klebsiella* relative abundance calculated previously (11) was also compared between mono- and mixed colonized patients (C). For (A-B), **, $p < 0.001$ using a Mann-Whitney t-test with $n \geq 18$. For (C), *, $p < 0.05$ with an n=171 and Mann-Whitney t-test.

Supplemental Tables

Supplemental Table 1: Applying *wzi*-Seq to a single *Klebsiella* strain sample

Pipeline	Reads After Filtering	Total Correct Reads	Total Incorrect Reads	Accurate Reads (%)	Total *wzi* types
error0.005	13,367	13,338	29	99.78	8
error0	6,181	6,179	2	99.96	3

Supplemental Table 2: The *wzi*-Seq method has high read accuracy with 5 and 12 *wzi* type mixtures

Sample Mixture	Pipeline	Average Reads After Filtering	Average Total Correct Reads	Average Total Incorrect Reads	Average Accurate Reads (%)	Average Total *wzi* Types
5 *wzi* Types	error0.005	9,932	9,854	78	99.3	13
	error0	4,322	4,319	3	99.9	6
12 *wzi* Types	error0.005	97,095	96,296	799	99.2	60
	error0	47,263	47,064	198	99.6	41

Supplemental Table 3: Summary table showing high precision between replicates for the 12 *wzi* type mixture

		error0.005		error0	
***wzi* Type**	**Expected Abundance (%)**	**Mean Abundance (%)**	**Standard Deviation**	**Mean Abundance (%)**	**Standard Deviation**
22	7.79	7.88	0.46	8.07	0.64
27	7.65	7.97	0.14	7.97	0.07
37	7.08	7.88	0.47	7.69	0.43
53	11.68	8.29	0.34	8.39	0.32
84	9.01	5.93	0.2	6	0.32
151	5.39	6.32	0.39	6.1	0.36
252	6.38	8.02	1.16	7.03	1.71
266	10.55	6.8	0.06	7.17	0.26
333	7.74	10.24	0.25	10.62	0.41
349	6.89	9.48	0.29	9.56	0.31
426	9.8	12.13	0.81	12.31	0.84
522	10.04	8.29	0.13	8.55	0.32

Supplemental Table 4: Contrived 30 and 58 *wzi* type mixtures have decreased accuracy with the error0.005 pipeline

Sample Mixture	**Pipeline**	**Reads After Filtering**	**Total Correct Reads**	**Total Incorrect Reads**	**Accurate Reads (%)**	**Total *wzi* Types**	**Correct *wzi* Types**
30 *wzi* Types	error0.005	58200	55562	2638	95.47	99	29
	error0	27446	27296	150	99.46	56	28
58 *wzi* Types	error0.005	39822	38637	1185	97.02	114	53
	error0	18836	18744	92	99.51	76	53

Supplemental Table 5: Illumina Sequencing of Rectal Swab Plate Sweeps Validates *wzi*-Seq Results

Sample	**Rectal Swab Status**	**Rectal Swab *wzi* Types (>1%)**	**Plate Sweep Status (*wzi*-Seq)**	**Plate Sweep *wzi*-Seq *wzi* Types (> 1%)**	**Plate Sweep Status (Illumina)**	**Plate Sweep Illumina *wzi* Types**
16461	Mono	wzi_427	Mono	wzi_427	Mono	wzi_427
16969	Mixed	wzi_527, wzi_27	Failed	Failed	Failed	Failed
17000	Mixed	wzi_273, wzi_300, wzi_84	Mixed	wzi_273, wzi_300, wzi_84	Mixed	wzi_273, wzi_300, wzi_84
17117	Mono	wzi_537	Mono	wzi_537	Mono	wzi_537
17665	Mixed	wzi_360, wzi_537, wzi_9, wzi_108, wzi_223, wzi_227, wzi_340	Mixed	wzi_360, wzi_537, wzi_9, wzi_108, wzi_223, wzi_227, wzi_340, wzi_158		
18599	Mono	wzi_19	Mono	wzi_19	Mono	wzi_19
18801	Mono	wzi_173	Mono	wzi_173	Mono	wzi_173
18922	Mixed	wzi_379, wzi_53	Mixed	wzi_379, wzi_53	Mixed	wzi_379, wzi_53

613 Supplemental Table 6: Results from final multivariable model of gut mono-colonization vs. mixed
614 colonization and the risk of subsequent *Klebsiella* infection

Variable	Odds Ratio	95% CI (Lower - Upper)	P Value
Age	0.989	0.961 – 1.02	0.417
Male Gender	0.835	0.421 – 1.64	0.602
Gut Colonization Month[1]	1.02	0.955 – 1.08	0.608
Mono-colonization	2.18	1.08 – 4.57	0.034
Prior Urinary Catheter	1.92	0.947 – 4.00	0.0733
Serum albumin ≥2.5 g/dL	0.353	0.167 – 0.731	0.00546
Serum albumin missing	0.382	0.0688 – 1.82	0.234

[1]Colonization month was coded from 0-18 chronologically for each month that gut colonized subjects were recruited.

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