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1 **SHORT TITLE**: MICROBIOME VS CULTURE TECHNIQUES

2

3 # **TITLE:** Comparison of microbiome and culture techniques for
4 determination of gastrointestinal microbial communities in
5 ceca of chickens

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23 # Abstract

24 The use of 16S next generation sequencing (NGS) technology to identify the relative
25 abundance of microbial communities have become the standard when studying the intestinal
26 microbiome. The increased use is due to the ability to identify a proportion of bacteria that
27 cannot be observed with culture-based methods. However, culture-based techniques are
28 acceptable to identify key bacterial groups, yet may grossly underestimate the microbial
29 community in question. Since there is limited research comparing NGS results to colony forming
30 units (CFU), the objective of this study was to compare total *Enterobacteriaceae* and lactic acid
31 bacteria (LAB) recovery with culture techniques (CFU/g ceca) to total number of reads from
32 operational taxonomic units (OTU) categorized as *Enterobacteriaceae* or LAB from Illumina
33 MiSeq platform from matched chick cecal samples at three and 10 days of age. Both CFU
34 recovery ($1.09 \times 10^9 \pm 2.42 \times 10^8$; $1.37 \times 10^8 \pm 5.57 \times 10^7$) and reads ($5460 \pm 1164$; 282 ± 163)
35 belonging to *Enterobacteriaceae* decreased by 10 days of age ($p \leq 0.001$). Similarly, LAB reads
36 decreased over time ($21{,}128 \pm 2262$; 6220 ± 817, respectively $p < 0.0001$). However, LAB CFU
37 recovery increased by 10 days ($1.18 \times 10^8 \pm 1.91 \times 10^7$; $1.62 \times 10^9 \pm 5.00 \times 10^8$, respectively $p <$
38 0.01). At three days the Pearson's correlation was -0.082 between CFU of culturable
39 *Enterobacteriaceae* to reads and culturable LAB CFU to reads at 0.097, showing no correlation
40 ($p = 0.606$, 0.551; respectively). By 10 days, no correlation of reads and CFU occurred with
41 *Enterobacteriaceae* ($r=-0.049$; p-value = 0.769) while with LAB the correlation was 0.290 ($p =$
42 0.066) at 10 days. The CFU may be appropriate to identify a few families that change due to
43 treatment or product. Without identifying viable cells to DNA recovered from NGS, there will
44 always be the question whether the reads within the binned OTU in the intestinal tract is
45 accurate.

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46 # Introduction

47 Ascertaining bacterial levels in samples is among one of the most fundamental
48 procedures in microbiology. Historically, measuring CFU on agar plates has been the most
49 common method, though quantitative PCR and other gene-based methods, such as next-
50 generation sequencing (NGS) have developed rapidly in the past 25 years [1–3]. The CFU
51 enumeration method has disadvantages that include miscounting due to clumping of cells and
52 inability to grow some types of bacteria in culture, commonly referred to as viable but non-
53 culturable species. Often with these species, microbiologists are unable to decipher and replicate
54 essential aspects of the gastrointestinal tract (GIT) environment within *in vitro* conditions. This
55 limits the ability to grow some species of bacteria, especially those that grow within micro-
56 environments of the GIT or those that are dependent on metabolic by-products and may require
57 co-culture with other species [4].

58 Recent advances in accessibility and affordability of NGS and metagenomics techniques
59 have increased its appeal to scientists that study microbial populations of the GIT because it has
60 provided an ability to measure bacteria beyond only those that could be detected by culture
61 methods and PCR. Metagenomic analysis requires reference-based identification and
62 quantification by aligning the reads However, there are no current standardized approaches for
63 these tools, as our understanding of the microbiome within the gut is at its infancy, especially as
64 scientists try to understand what is or is not biologically relevant [5]. Thus, it is essential to
65 understand variations of the microbiome in response to dietary amendments, additive insertion,
66 litter management, etc. in relation to gut health [6–8]. Abundance taxonomy does not supply the
67 complete picture required to understand how any treatment impacts the health of the GIT or the
68 causation of dysbacteriosis, known to have detrimental effects on the host, because it does not

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69 include host responses, nor does it present insight into phenotypic changes within the
70 microbiome [9–11]. However, there may be circumstances under which culture methodologies
71 are sufficient, such as measuring changes in the levels of *Salmonella* within ceca. Though,
72 culture-based techniques of digesta typically allow for differentiation at the genera level, full
73 identification often requires multiple biochemical tests, and still often relies upon gene-based
74 techniques to confirm identification. The objective of this study was to compare culture recovery
75 of bacteria on MacConkey agar (MAC), for recovery of Gram-negative bacteria, and De Man,
76 Rogosa and Sharpe agar (MRS) for recovery of lactic acid bacteria (LAB), to paired NGS
77 microbiome detection of bacteria within the ceca of chickens. The goal of these comparisons was
78 to determine differences and similarities of these methodologies in order to help scientists assess
79 appropriate methods for their research goals while studying microbiota.

80 Materials and Methods

81 Embryo incubation and animal housing

82 Fertile broiler eggs were obtained from a local hatchery and placed in a single-stage
83 incubator (Natureform Inc. Jacksonville, FL) until 18 embryonic days (18ED), then were
84 randomly placed into one of 12 benchtop hatchers (Hova-Bator model 1602N, Savannah, GA,
85 USA), which had been disinfected with 10% bleach prior to use. Up to 30 eggs were placed each
86 hatcher to avoid overcrowding.

87 Immediately post-hatch, 128 chicks were placed into brooder battery cages that had wire
88 floors covered with paper to encourage exposure of chicks to fecal material, and the paper was
89 allowed to disintegrate naturally for the duration of the experiment. Age-appropriate ambient
90 temperature was maintained with 24 hour lighting during the first seven days, followed by one

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91 hour of darkness through 10 days. Chicks has *ad libitum* access to a standard corn soy diet and
92 water [12]. All activities were conducted under approved Institutional Animal Care and Use
93 Committee protocols.

94 Sample Collection

95 At three days post hatch, 80 chicks were removed for culture and microbiome analysis,
96 with two chicks pooled to create a single sample (n=40). Since chicks were substantially larger
97 with more digesta content, at 10 days, 45 chicks were sampled for both microbiome and culture
98 analysis. Chicks were killed via cervical dislocation and all samples were aseptically collected
99 post mortem. From each chick, a cecum was designated for culture-based recovery of bacteria,
100 and the other for microbiome NGS.

101 Culture Isolation and Enumeration

102 Cecal samples were placed into sterile bags and pulverized with a rubber mallet to
103 expose contents. Samples were then weighed and 0.9% sterile saline added to make a five-fold
104 dilution, after which serial 10-fold dilutions were made for plating on MAC (VWR, Suwanee,
105 GA, USA) and MRS (Difco™ Lactobacilli MRS AGAR VWR, Suwanee, GA, USA). All agar
106 plates were incubated for up to 24 h at 37ºC in an aerobic environment. Enumeration values from
107 each selective medium for each sample were calculated to the original CFU/gram of ceca.

108 DNA Isolation and Library Preparation

109 Ceca for DNA isolation were placed in 1.5mL tubes and flash-frozen in liquid nitrogen at
110 the time of collection, then stored at -80 °C until further use. The Qiagen stool kit (Qiagen,
111 Valencia, CA, USA) was utilized for DNA isolation following methods of Yu and Morrison
112 [13]. Quantification of extracted DNA was completed with a Synergy HTX multi-mode plate

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113 reader (BioTek U.S., Winooski, VT, USA) and 5ng of DNA from each sample were randomly
114 aliquoted onto a 96-well plate. All plates included known bacterial isolates as a positive control,
115 plus a well with milliQ water as a negative control. MiSeq library preparation and 2 x 300
116 paired-end sequencing (Illumina, San Diego, CA, USA) was performed by the Ohio State
117 University Molecular and Cellular Imaging Center (OSU MCIC). Primers amplified the V4-V5
118 region of the 16S rRNA gene (515F: GTGYCAGCMGCCGCGGTAA, 806R:
119 GGACTACHVGGGTWTCTAAT; Ballou et al., 2016).

120 ## 16S Sequence Data Analysis

121 After quality control from sequencing a total of 36,437,234 Illumina MiSeq reads were
122 generated, and represented the prepared sequencing libraries for this study. The raw FASTQ files
123 were de-multiplexed, primers and spacers removed and quality-filtered with QIIME 1.9.0 [15].
124 Quality filter variables included a minimum length of 200 base pairs, average quality score of 20,
125 no barcode errors, reads were removed if there were two nucleotide mismatched, and chimeras
126 were removed. Similar sequences were clustered together using the open-reference operational
127 taxonomic unit (OTU) picking protocol, sequences were grouped into OTUs based on 97%
128 sequence identity using the Ribosomal Database Project, with a minimum of 1,000 reads for each
129 sample. Singleton and doubleton OTUs were removed due to potential sequencing errors or non-
130 significant microbes. After initial quality processing, a total of 339 OTUs were identified.
131 Represented sequences from each OTU were picked and assigned taxonomy and sequences with
132 high identity (> 97%), and were clustered in the same OTU. The sequence coverage was
133 normalized across all samples with a $\log_{10}$ transformation. Taxonomic assignments were
134 generated using QIIME and R-studio (RStudio, Inc.). Taxonomic affiliations of sequences or
135 reads classified under family *Enterobacteriaceae* or order Lactobacillales were counted which

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136 indicated the total number of reads within each binned OTU identified as either
137 *Enterobacteriaceae* family or Lactobacillales order. These classifications were chosen because
138 they primarily cover the types of bacteria recovered on MAC and MRS.

139 Statistical Analysis

140 A Student's t-test was performed to identify if there were changes in the number of reads
141 and CFU/g ceca of *Enterobacteriaceae* and LAB over time, three days and 10 days (JMP 12.2.0,
142 SAS Institute Inc). Observational jitter boxplots were constructed to visualize the distribution of
143 cecal CFU/g of ceca for every sample from the MAC and MRS, as well as the number of reads
144 belonging to OTUs categorized as *Enterobacteriaceae* or LAB on each day of collections
145 (RStudio, Inc.). To evaluate the relationship between number of reads and CFU, a simple linear
146 regression model was developed based on the equation:

147
$$Y = \beta_0 + \beta_1 X$$

148 With β_0 representing the intercept, and β_1 representing the slope in relation to the corresponding
149 to X or the CFU/g ceca count. The data were fitted to a straight line by the least squares method,
150 which assessed the relationship in the model using the coefficient of determination, R^2 and the
151 relationship between the two variables using Pearson's correlation coefficient ρ, and a p-value of
152 ≤ 0.05 were deemed significant (JMP 12.2.0, SAS Institute Inc).

153 Results and Discussion

154 The use of culture-independent methods to characterize microbial communities has
155 increased astronomically over the past few years, both due to major advances in NGS and ability
156 to identify a large proportion of bacteria that are difficult or impossible to observe with culture

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157 based techniques [16]. Both culture dependent and culture independent methods have a role in
158 microbiome studies; however, depending on what is being assessed one method may be better
159 than the other or they should be used together. This research study compared culture and NGS
160 methods to elucidate differences and similarities of the methods to allow for better use of these
161 technologies in avian microbiome research.

162 Total reads from binned OTU *Enterobacteriaceae* and *Lactobacillales* at three days of
163 age were significantly ($p < 0.0001$) higher (1,063,533) compared to the total number of reads
164 (266,582) present in the ceca at 10 days of age. The genus *Pseudomonas* can grow on MAC and
165 is described as colorless [17]. *Pseudomonas* was not present with culture methods or microbiome
166 analysis, thus the family *Enterobacteriaceae* alone was analyzed. At the neonatal stage of any
167 mammalian or avian species, the diversity and abundance of microflora is generally low and as
168 the animal ages this diversity and abundance increases. The increased intestinal diversity
169 inadvertently decreases the relative abundance of predominantly aerobic or facultative anaerobic
170 bacteria including the *Lactobacillales* order and *Enterobacteriaceae* family [14,18–20]. In this
171 study, the *Enterobacteriaceae* populations significantly decreased by 10 days of age according to
172 OTU counts (5460 ± 1164 ; 282 ± 163, $p < 0.0001$) and CFU recovery ($1.09 \times 10^9 \pm 2.42 \times 10^8$;
173 $1.37 \times 10^8 \pm 5.57 \times 10^7$, $p < 0.001$), respectively. Similarly, LAB OTU counts showed a decrease
174 over time from three to 10 days of age ($21{,}128 \pm 2262$ at three days of age; 6220 ± 817 at 10
175 days of age, $p < 0.0001$). However, LAB recovery significantly increased in CFU from three to
176 10 days of age ($1.18 \times 10^8 \pm 1.91 \times 10^7$ at three days of age; $1.62 \times 10^9 \pm 5.00 \times 10^8$ at 10 days of age,
177 $p < 0.01$). Figure 1 shows the differences seen in relative abundance from three to 10 days of
178 age. The relationship between CFU and OTU did not correlate at three days of age (Fig 1A). At
179 this time, the total recovered *Enterobacteriaceae* on MAC represented 64.8% of the total relative

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180 CFU/g and the remaining 35.2% belonged to LAB ($p < 0.001$); however, at the OTU level,
181 *Enterobacteriaceae* represented 22.2% of the total relative reads and the remaining 77.8%
182 belonged to the LAB ($p < 0.01$). By 10 days of age, the proportions were not significantly
183 different in the culture and NGS methods with *Enterobacteriaceae* represented at 8.1% and 4.6%
184 of the CFU/g ceca and OTU counts, respectively, while LABs accounted for 91.9% and 95.4%
185 of the CFU and OTU counts, respectively (Fig 1B). The CFU/g ceca and total number of reads
186 data were analyzed separately to provide the relationship of bacterial presence over time with the
187 two types of techniques. However, because CFU and reads from binned OTUs do not provide the
188 same units of measure, they could not be directly analyzed for statistical relevance. The
189 differences between CFU and OTU at three days of age in Fig 1A may reflect the rapid turnover
190 of bacteria that could be dormant, non-viable or non-culturable.

191

192 **Fig. 1. Proportion of *Enterobacteriaceae* and LAB CFU/g ceca or Reads from respective**
193 ***Enterobacteriaceae* and LAB OTU.**
194 **(**A) three days (n=40); (B) 10 days (n=45) post-hatch. The p-value indicates comparison of
195 *Enterobacteriaceae* and LAB within methodology (CFU/gram ceca or total reads from binned
196 OTU) and time of collection, three days and 10 days.

197

198 The retention time of feed in the small intestine has been determined to be approximately
199 three hours, with the ileum being the bottleneck as there are a reduced amount of digestible
200 components in this space that can slow the flow rate [21]. Between four and 10 days of age,
201 broilers have been observed to experience a rapid increase in feed intake with a parallel decrease

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202 in passage rate, which may be partially due to quicker absorption of amino-acid derived nitrogen
203 and fatty acids [22]. This change in digesta flow may account for nutrient availability for bacteria
204 at three days of age. For example, *Lactobacillus* are a complex organism that require certain
205 amino acids, vitamins and sugars to thrive [23].

206 Results in this experiment show that reads from OTUs do not provide the same answers
207 at CFU recovery for particular times of collection. Rubinelli et al (2017) found the inclusion of
208 acidifiers such as sodium bisulfate (SBS) were associated with decreased recovery of *Salmonella*
209 from *in vitro* spiked chicken cecal contents at 48 hours post incubation; however, the relative
210 abundance showed no difference in microbial composition after 24 hours of incubation and no
211 difference of the abundance of Gammaproteobacteria class or *Enterobacteriaceae* family, in
212 which *Salmonella* belongs [24]. For this study, the viable bacteria recovered on MAC ranged
213 from 3.50×10^7 to 8.00×10^9 CFU/g ceca at three days of age (Fig 2A).

214

215 **Fig 2. Jitter boxplot representing total CFU/g ceca or Reads from Enterobacteriaceae or**
216 **LAB genera at three days.**
217 (A) CFU/g ceca from MacConkeys agar (MAC), Man Rogosa and Sharpe agar (MRS), (B) total
218 number of reads from the *Enterobacteriaceae* family or lactic acid producing bacteria (LAB)
219 order Lactobacillales OTUs from cecal samples (n=40) at three days of age. Each dot represents
220 a single sample. The solid black line represents the mean. The box outline represents the ±
221 standard deviation

222

223 Conversely, *Enterobacteriaceae* OTU number of reads had a distribution of 63 to 41,741
224 total reads at 3 days of age (Fig 2B). By 10 days of age, *Enterobacteriaceae* MAC recovery

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225 range was 2.50×10^5 to 2.00×10^9 CFU/g ceca (Fig 3A) and number of reads had a range of 0 to
226 6,327 OTU counts (Fig 3B). The LAB recovery distribution at 3 days of age was 0.00 to
227 5.80×10^8 CFU/g on MRS (Fig 2A) whereas the number of reads from LAB OTU ranged from
228 1,105 - 70,087 (Fig 2B). By 10 days of age, the distribution of LAB recovered on MRS was
229 1.30×10^7 to 1.5×10^{10} CFU/g ceca (Figure 3A) and the OTU counts had a range of 207 to 21,105
230 (Fig 3B). The results showed no correlation between total reads and CFU counts for
231 *Enterobacteriaceae* (ρ =-0.082; p-value = 0.606; Fig 4A) or LAB (ρ = 0.097; p-value = 0.551;
232 Fig 4B) and no correlation at 3 days of age (ρ = -0.049; p-value = 0.769; Fig 5A) or 10 days or
233 age (ρ = 0.290; p-value = 0.066; Fig 5B). Currently, there are no standard methods for
234 comparison of CFU and OTU data. These figures (Fig 1-5) illustrate the different patterns within
235 the same day and type of bacteria recovered.

236

237 **Fig. 3. Jitter boxplot representing total CFU/g ceca or Reads from Enterobacteriaceae or**
238 **LAB genera at ten days.**

239 (A) CFU/g ceca from MacConkeys agar (MAC), Man Rogosa and Sharpe agar (MRS), (B) total
240 number of reads from the *Enterobacteriaceae* family or lactic acid producing bacteria (LAB)
241 order Lactobacillales OTUs from cecal samples (n=45) at three days of age. Each dot represents
242 a single sample. The solid black line represents the mean. The box outline represents the ±
243 standard deviation

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247 **Fig. 4. Dot plot depicting the relationship of total number of reads and CFU/g ceca of A)**
248 ***Enterobacteriaceae* and B) LAB from three day-old chicks (n=40).**
249 The black line represents the fitted regression linear regression line with its corresponding
250 equation and correlation of determination (R^2), Pearson's correlation coefficient between number
251 of reads and CFU (ρ) and the p-value
252

253 **Figure 5. Dot plot depicting the relationship of total number of reads and CFU/g ceca of A)**
254 ***Enterobacteriaceae* and B) LAB from ceca of 10 day old chicks (n=45).**
255 The black line represents the fitted regression linear regression line with its corresponding
256 equation and correlation of determination (R^2), Pearson's correlation coefficient between number
257 of reads and CFU (ρ) and the p-value
258

259 Quantifying microbiota by 16S rRNA have not been able to determine associations with
260 microorganisms in an active or potentially active state because the results include active as well
261 as dormant, dead and quiescent bacteria which are present in all microbial samples [25]. This
262 lack of ability to detect DNA from only active cells beg the question whether the number of
263 reads within the binned OTUs in any biological system are accurate representations of active
264 microbial communities. In this study, the total relative abundance of *Enterobacteriaceae* and
265 LABs were different between three and 10 days of age (three days of age: 4.24%, 39.37%; 10
266 days of age: 21.74%, 1.91%, respectively; data not shown). In general, the alpha diversity (i.e.
267 number of species present within a sample) is increased as the animal ages and it is unlikely that
268 culture based recovery will be able to reflect this change since a wide variety of media would be
269 required and viable but non-culturable species would not be detected [14,20,26,27]. Many

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270 *Lactobacillales* are facultative anaerobes but some species, such as *Lactobacillus fermennti*, are
271 slow growing and other genera, such as *Leuconostoc* and *Pediococcus*, may not grow as
272 fastidiously as *Lactobacillus* on MRS agar [28].

273 The bacterial groups recovered from the *Enterobacteriaceae* family included the genus
274 *Citrobacter*, *Klebsiella* (i.e. *Citrobacter* 2), *Proteus* and *Trabulsiella*. The total OTUs present in
275 the LAB or *Lactobacillales* order included the genus *Enterococcus*, *Lactobacillus*, *Pediococcus*,
276 *Streptococcus* and *Leuconostoc*. A common marker that researchers examine in improved gut
277 health is an increase LAB. Many bacteria produce lactic acid as an end-product, a LAB primary
278 metabolic end product is lactic acid by glucose metabolism. The LAB are functionally comprised
279 of catalase negative and Gram-positive bacteria and the group typically falls under the order
280 *Lactobacillales* [29]. The LABs include the genus *Lactobacillus*, *Enterococcus*, *Lactococcus*,
281 *Leuconstoc*, *Pediococcus* and *Streptococcus*. Choosing known families or orders of bacteria such
282 as *Enterobacteriaceae* and *Lactobacillales* to monitor with culture based techniques may be
283 sufficient to answer questions related to efficacy of a treatment since the counts are of live
284 culturable bacteria and not DNA.

285 The term CFU is based on the idea that microbial colonies counted on agar began as a
286 single cell or aggregates of several cells [30]. In DNA-based sequencing techniques, the number
287 of taxonomic units that have been identified in a given sample are deemed as OTU. This unit is
288 defined based on sequence similarity [31]. For this similarity, 97% of the DNA sequence of the
289 bacterial 16S rRNA gene is commonly used to define a taxonomic genus and 98-99% for species
290 [31]. This level of sequence resemblance is believed to compare the common species but this can
291 be based on morphology, physiology and other characteristics of organisms, so the terms species
292 and OTU may not be fully comparable [31].

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293 Some researchers support their 16S rRNA sequencing with previous CFU findings,
294 particularly for a single bacterial genus [32,33]. However, a consequence of the immense
295 information that microbiome-based analysis provides is there may be more difficulty to find
296 statistically different data compared to CFU counts [34]. Approximately 20% of bacteria in the
297 gastrointestinal tract (GIT) of a chicken are culturable so CFU counts alone will provide an
298 underestimation of the total microbiota present [35]. Analysis of the microbial composition is not
299 necessarily limited by technical abilities, but rather inconsistencies throughout the literature and
300 experiments, including bias generated by PCR, as well as sequence coverage and length, which
301 greatly alter the taxonomic information [25,36–39].

302 The increased high throughput abilities of NGS has provided researchers with invaluable
303 information regarding the presence or absence of total bacteria in a microbial community.
304 However, this technology does have limitations. This results from this study showed that
305 culturing and NGS techniques demonstrated little to no correlation on the presence or absence of
306 two groups of bacteria that were tested. While CFU/g ceca and OTU provide different results,
307 each technique may be appropriate for different situations. For example, CFU would be
308 acceptable when the objective is to identify culturable bacteria or recovery of a single or few
309 species of known bacteria. Conversely, NGS is beneficial to identify overall community changes
310 in situations where global DNA content may provide a more complete answer. Each
311 methodology of quantification may yield different results so care should be taken to select the
312 correct microbiological techniques to test a hypothesis in order to avoid drawing conclusions that
313 are not represented by the type of data analyzed.

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314 # Acknowledgements

315 The authors would like to thank the farm personnel at the OARDC Poultry Research Farm
316 including Keith Patterson for providing the eggs. Kendal Searer is greatly acknowledged for her
317 technical assistance as well as Saranga Wijerante, MS for his bioinformatics guidance. Lastly,
318 the authors would like to acknowledge and thank the OARDC Research Enhancement
319 Competitive Grants Program (SEEDS) #2016035 for providing the funding of this research

320 # Author Contributions

321 Conceived and designed the experiments: KMW, LRB. Participated in sample collections:
322 KMW, WRB, AFD, KMC. Analyzed the data: KMW, XS. Contributed to
323 reagents/materials/analysis tools: KMW, WRB, XS. Wrote the paper: KMW, LRB. Critical
324 review of manuscript: LRB and XS.

325 # References

326 1. Monod J. The Growth of Bacterial Cultures. Annual Review of Microbiology. 1949;3: 371–
327 394. doi:10.1146/annurev.mi.03.100149.002103

328 2. van Dijk EL, Auger H, Jaszczyszyn Y, Thermes C. Ten years of next-generation
329 sequencing technology. Trends in Genetics. 2014;30: 418–426.
330 doi:10.1016/j.tig.2014.07.001

331 3. Hagemann IS. Chapter 1 - Overview of Technical Aspects and Chemistries of Next-
332 Generation Sequencing. In: Kulkarni S, Pfeifer J, editors. Clinical Genomics. Boston:
333 Academic Press; 2015. pp. 3–19. doi:10.1016/B978-0-12-404748-8.00001-0

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334 4. Stewart EJ. Growing Unculturable Bacteria. J Bacteriol. 2012;194: 4151–4160.
335 doi:10.1128/JB.00345-12

336 5. He Y, Caporaso JG, Jiang X-T, Sheng H-F, Huse SM, Rideout JR, et al. Stability of
337 operational taxonomic units: an important but neglected property for analyzing microbial
338 diversity. Microbiom. 2015;3: 20. doi:10.1186/s40168-015-0081-x

339 6. Park SH, Perrotta A, Hanning I, Diaz-Sanchez S, Pendleton S, Alm E, et al. Pasture flock
340 chicken cecal microbiome responses to prebiotics and plum fiber feed amendments. Poult
341 Sci. 2017;96: 1820–1830. doi:10.3382/ps/pew441

342 7. Roto SM, Kwon YM, Ricke SC. Applications of *In Ovo* Technique for the Optimal
343 Development of the Gastrointestinal Tract and the Potential Influence on the Establishment
344 of Its Microbiome in Poultry. Front Vet Sci. 2016;3. doi:10.3389/fvets.2016.00063

345 8. Stanley D, Denman SE, Hughes RJ, Geier MS, Crowley TM, Chen H, et al. Intestinal
346 microbiota associated with differential feed conversion efficiency in chickens. Appl
347 Microbiol Biotechnol. 2012;96: 1361–1369. doi:10.1007/s00253-011-3847-5

348 9. Teirlynck E, Gussem MDE, Dewulf J, Haesebrouck F, Ducatelle R, Immerseel FV.
349 Morphometric evaluation of "dysbacteriosis" in broilers. Avian Pathology. 2011;40: 139–
350 144. doi:10.1080/03079457.2010.543414

351 10. Belkaid Y, Hand T. Role of the Microbiota in Immunity and inflammation. Cell. 2014;157:
352 121–141. doi:10.1016/j.cell.2014.03.011

bioRxiv preprint doi: https://doi.org/10.1101/494781; this version posted December 12, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

353 11. Lan Lin, Jianqiong Zhang. Role of intestinal microbiota and metabolites on gut homeostasis
354 and human diseases. BMC Immunology. 2017;18: 1–25. doi:10.1186/s12865-016-0187-3

355 12. Nutrient Requirements of Poultry [Internet]. Washington, D.C.: National Academies Press;
356 1994. doi:10.17226/2114

357 13. Yu Z, Morrison M. Improved extraction of PCR-quality community DNA from digesta and
358 fecal samples. BioTechniques. 2004;36: 808–812.

359 14. Ballou AL, Ali RA, Mendoza MA, Ellis JC, Hassan HM, Croom WJ, et al. Development of
360 the Chick Microbiome: How Early Exposure Influences Future Microbial Diversity. Front
361 Vet Sci. 2016;3: 1–12. doi:10.3389/fvets.2016.00002

362 15. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, et al. Ultra-
363 high-throughput microbial community analysis on the Illumina HiSeq and MiSeq
364 platforms. ISME J. 2012;6: 1621–1624. doi:10.1038/ismej.2012.8

365 16. Zapka C, Leff J, Henley J, Tittl J, De Nardo E, Butler M, et al. Comparison of Standard
366 Culture-Based Method to Culture-Independent Method for Evaluation of Hygiene Effects
367 on the Hand Microbiome. mBio. 2017;8. doi:10.1128/mBio.00093-17

368 17. Abbas M, Emonet S, Köhler T, Renzi G, van Delden C, Schrenzel J, et al. Ecthyma
369 Gangrenosum: *Escherichia coli* or *Pseudomonas aeruginosa*? Front Microbiol. 2017;8.
370 doi:10.3389/fmicb.2017.00953

bioRxiv preprint doi: https://doi.org/10.1101/494781; this version posted December 12, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

371 18. Palmer C, Bik EM, DiGiulio DB, Relman DA, Brown PO. Development of the Human
372 Infant Intestinal Microbiota. PLOS Biology. 2007;5: e177.
373 doi:10.1371/journal.pbio.0050177

374 19. Houghteling PD, Walker WA. Why is initial bacterial colonization of the intestine
375 important to the infant's and child's health? J Pediatr Gastroenterol Nutr. 2015;60: 294–
376 307. doi:10.1097/MPG.0000000000000597

377 20. Awad WA, Mann E, Dzieciol M, Hess C, Schmitz-Esser S, Wagner M, et al. Age-Related
378 Differences in the Luminal and Mucosa-Associated Gut Microbiome of Broiler Chickens
379 and Shifts Associated with *Campylobacter jejuni* Infection. Front Cell Infect Microbiol.
380 2016;6: 1–17. doi:10.3389/fcimb.2016.00154

381 21. Liu JD, Secrest SA, Fowler J. Computed tomographic precision rate-of-passage assay
382 without a fasting period in broilers: More precise foundation for targeting the releasing time
383 of encapsulated products. Livestock Science. 2017;200: 60–63.
384 doi:10.1016/j.livsci.2017.04.006

385 22. Noy Y, Sklan D. Digestion and absorption in the young chick. Poultry Science. 1995; 366–
386 373.

387 23. Apajalahti J, Vienola K. Interaction between chicken intestinal microbiota and protein
388 digestion. Animal Feed Science and Technology. 2016;221, Part B: 323–330.
389 doi:10.1016/j.anifeedsci.2016.05.004

390 24. Rubinelli PM, Kim SA, Park SH, Roto SM, Ricke SC. Sodium bisulfate and a sodium
391 bisulfate/tannin mixture decreases pH when added to an in vitro incubated poultry cecal or

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392 fecal contents while reducing *Salmonella* Typhimurium marker strain survival and altering
393 the microbiome. Journal of Environmental Science and Health, Part B. 2017;52: 607–615.
394 doi:10.1080/03601234.2017.1316159

395 25. Rojo D, Méndez-García C, Raczkowska BA, Bargiela R, Moya A, Ferrer M, et al.
396 Exploring the human microbiome from multiple perspectives: factors altering its
397 composition and function. FEMS Microbiol Rev. 2017;41: 453–478.
398 doi:10.1093/femsre/fuw046

399 26. Sergeant MJ, Constantinidou C, Cogan TA, Bedford MR, Penn CW, Pallen MJ. Extensive
400 Microbial and Functional Diversity within the Chicken Cecal Microbiome. PLOS ONE.
401 2014;9: e91941. doi:10.1371/journal.pone.0091941

402 27. Oakley BB, Kogut MH. Spatial and Temporal Changes in the Broiler Chicken Cecal and
403 Fecal Microbiomes and Correlations of Bacterial Taxa with Cytokine Gene Expression.
404 Front Vet Sci. 2016;3: 1–12. doi:10.3389/fvets.2016.00011

405 28. Schillinger U, Lücke F-K. Identification of lactobacilli from meat and meat products. Food
406 Microbiology. 1987;4: 199–208. doi:10.1016/0740-0020(87)90002-5

407 29. Salvetti E, Torriani S, Felis GE. The Genus Lactobacillus: A Taxonomic Update. Pro
408 Antimicro Prot. 2012;4: 217–226. doi:10.1007/s12602-012-9117-8

409 30. Ben-Jacob E, Schochet O, Tenenbaum A, Cohen I, Czirók A, Vicsek T. Generic modelling
410 of cooperative growth patterns in bacterial colonies. Letters to Nature. 1994;368: 46–49.
411 doi:10.1038/368046a0

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412 31. Rosselló-Mora R, Amann R. The species concept for prokaryotes. FEMS Microbiol Rev.
413 2001;25: 39–67. doi:10.1111/j.1574-6976.2001.tb00571.x

414 32. Schlaberg R, Chiu CY, Miller S, Procop GW, Weinstock G. Validation of Metagenomic
415 Next-Generation Sequencing Tests for Universal Pathogen Detection. Archives of
416 Pathology & Laboratory Medicine. 2017;141: 776–786. doi:10.5858/arpa.2016-0539-RA

417 33. Sun X, Winglee K, Gharaibeh RZ, Gauthier J, He Z, Tripathi P, et al. Microbiota-derived
418 Metabolic Factors Reduce Campylobacteriosis in Mice. Gastroentero. 2018;
419 doi:10.1053/j.gastro.2018.01.042

420 34. Olsen R, Kudirkiene E, Thøfner I, Pors S, Karlskov-Mortensen P, Li L, et al. Impact of egg
421 disinfection of hatching eggs on the eggshell microbiome and bacterial load. Poult Sci.
422 2017;96: 3901–3911. doi:10.3382/ps/pex182

423 35. Gaskins HR, Collier CT, Anderson DB. Antibiotics as growth promotants: mode of action.
424 Animal Biotechnology. 2002;13: 29–42.

425 36. Youssef N, Sheik CS, Krumholz LR, Najar FZ, Roe BA, Elshahed MS. Comparison of
426 Species Richness Estimates Obtained Using Nearly Complete Fragments and Simulated
427 Pyrosequencing-Generated Fragments in 16S rRNA Gene-Based Environmental Surveys.
428 Appl Environ Microbiol. 2009;75: 5227–5236. doi:10.1128/AEM.00592-09

429 37. Stanley D, Hughes RJ, Moore RJ. Microbiota of the chicken gastrointestinal tract: influence
430 on health, productivity and disease. Appl Microbiol Biotechnol. 2014;98: 4301–4310.
431 doi:10.1007/s00253-014-5646-2

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432 38. Keller A, Horn H, Förster F, Schultz J. Computational integration of genomic traits into
433 16S rDNA microbiota sequencing studies. Gene. 2014;549: 186–191.
434 doi:10.1016/j.gene.2014.07.066

435 39. Jovel J, Patterson J, Wang W, Hotte N, O'Keefe S, Mitchel T, et al. Characterization of the
436 Gut Microbiome Using 16S or Shotgun Metagenomics. Front Microbiol. 2016;7.
437 doi:10.3389/fmicb.2016.00459

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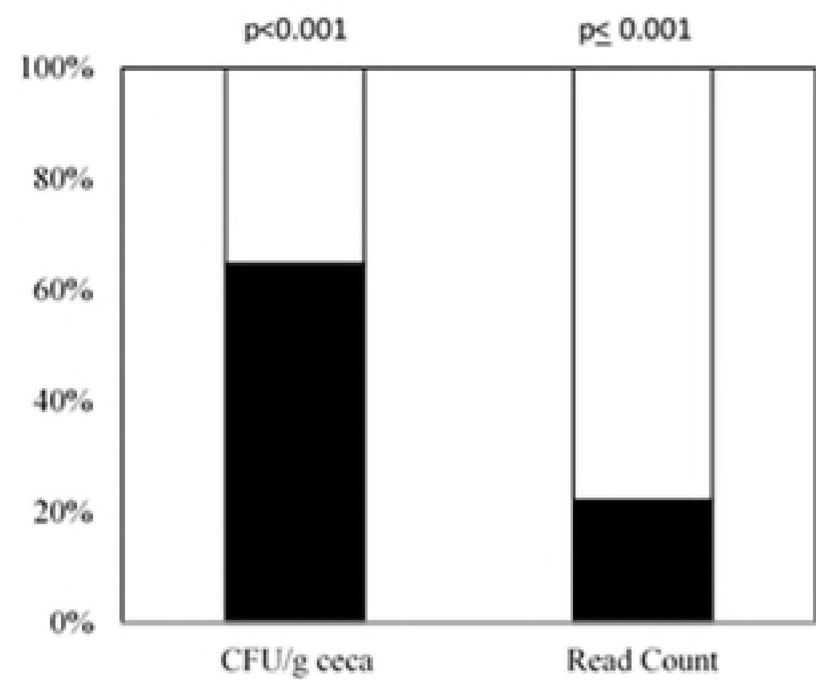


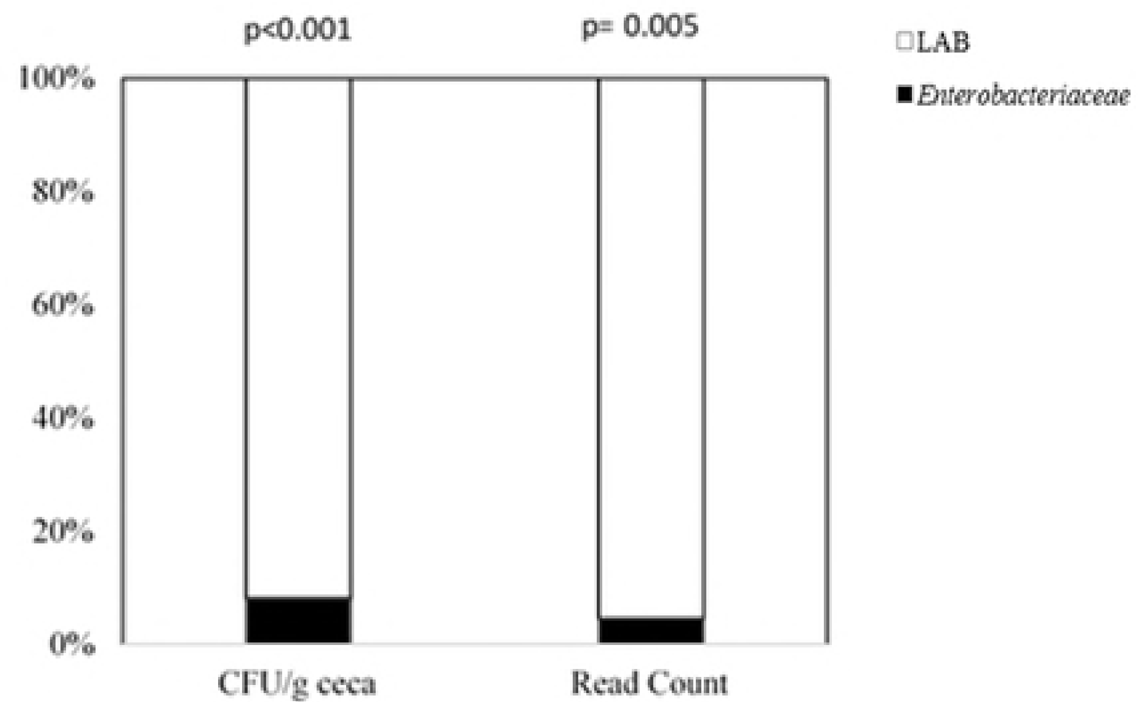
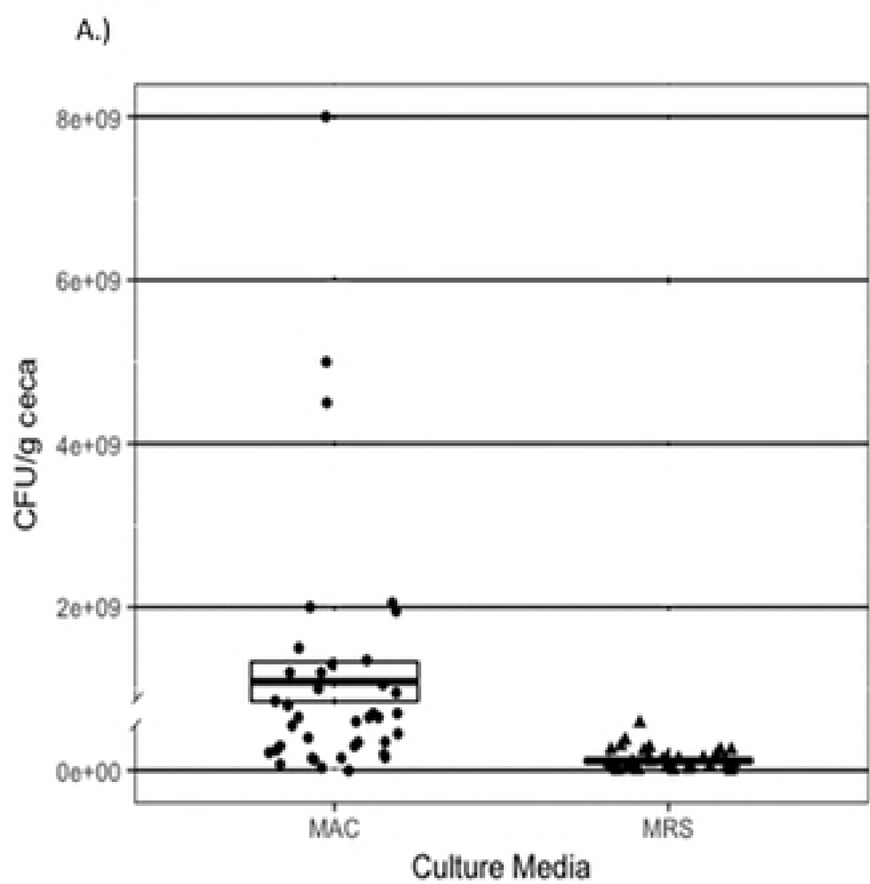


Figure 1



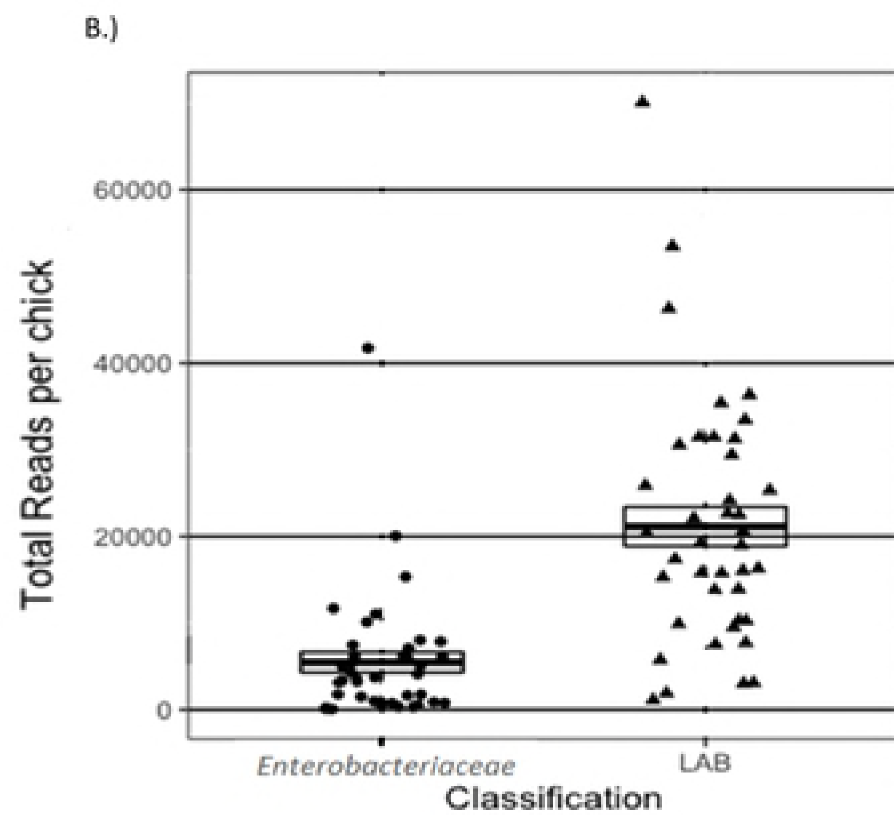


Figure 2

A.)

1.5e+10
1.0e+10
5.0e+09
0.0e+00

CFU/g ceca

MAC
MRS

Culture Media

B.)

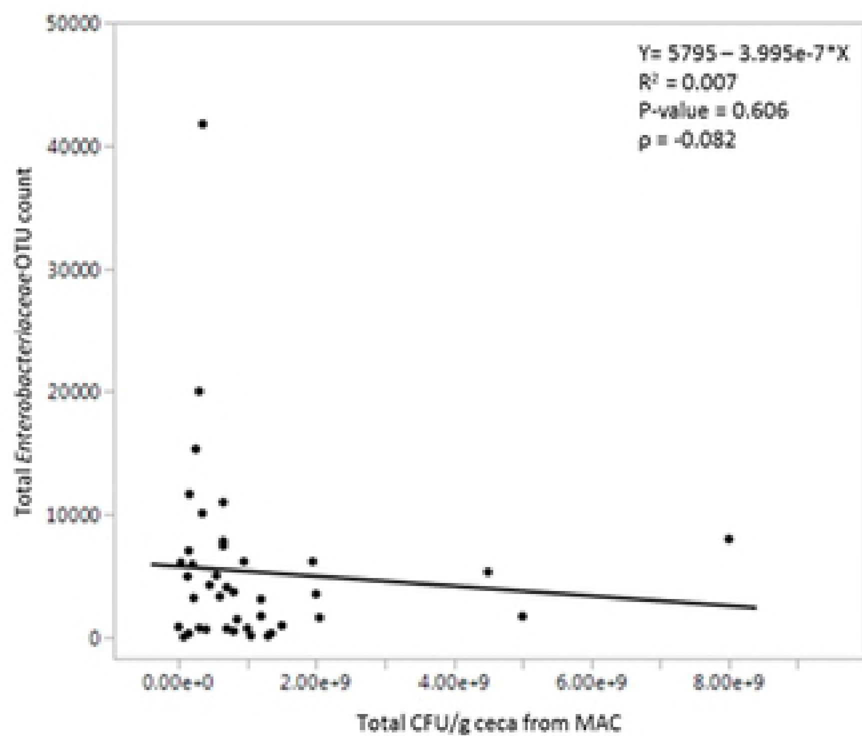
20000
15000
10000
5000
0

Total Reads per chick

Enterobacteriaceae
LAB

Classification

Figure 3



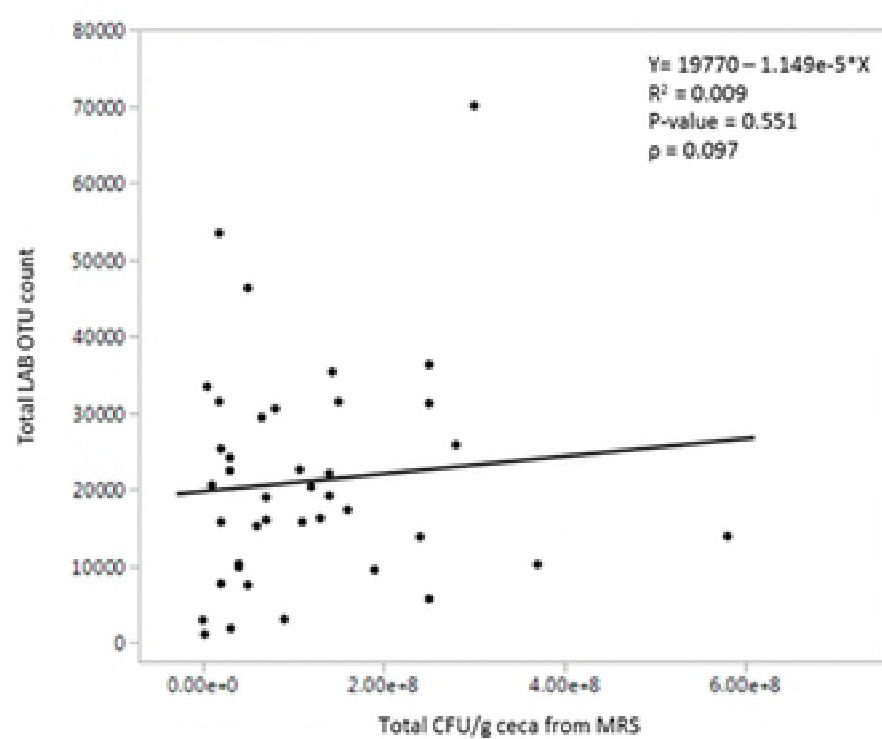
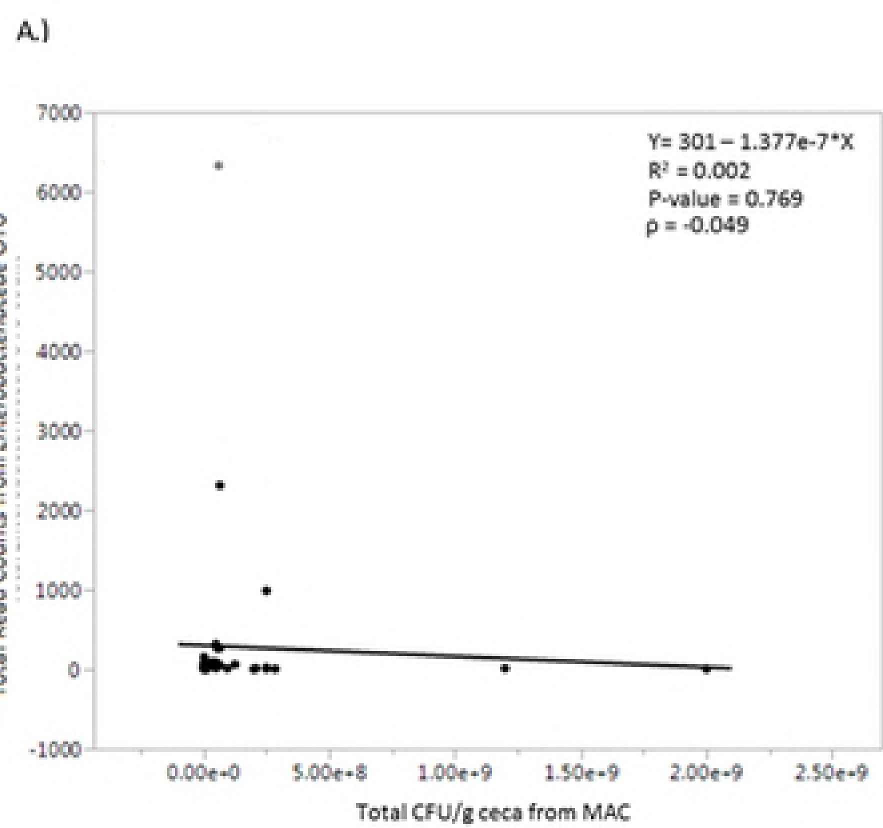


Figure 4



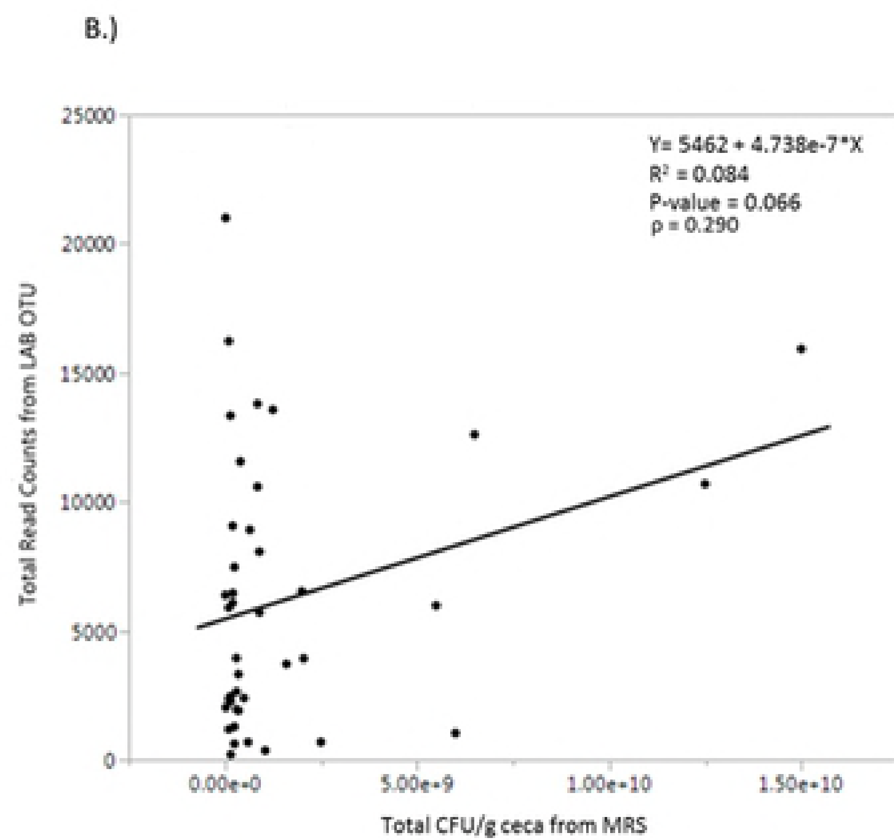


Figure 5