1   **Cannabis microbiome sequencing reveals several mycotoxic fungi native to**
2   **dispensary grade cannabis flowers**
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8
9   **Abstract**
10   The Center for Disease Control estimates 128,000 people in the U.S. are hospitalized
11   annually due to food borne illnesses. This has created a demand for food safety testing
12   targeting the detection of pathogenic mold and bacteria on agricultural products. This risk
13   extends to medical Cannabis and is of particular concern with inhaled, vaporized and
14   even concentrated Cannabis products. As a result, third party microbial testing has
15   become a regulatory requirement in the medical and recreational Cannabis markets, yet
16   knowledge of the Cannabis microbiome is limited. Here we describe the first next
17   generation sequencing survey of the microbial communities found in dispensary based
18   Cannabis flowers and demonstrate the limitations in the culture based regulations that are
19   being superimposed from the food industry.
20
21   **Introduction**
22   Many states in the U.S. are crafting regulations for microbial detection on cannabis in
23   absence of any comprehensive survey of Cannabis microbiomes. A few of these
24   regulations are inducing growers to "heat kill" or pasteurize *Cannabis* flowers to lower
25   microbial content. While this is a harmless suggestion, we must remain aware of how
26   these drying techniques often create false negatives in culture based safety tests used to
27   monitor colony-forming units (CFU). Even though pasteurization may be effective at
28   sterilizing some of the microbial content, it does not eliminate various pathogenic toxins
29   or spores. Aspergillus spores and mycotoxins are known to resist pasteurization [1, 2].
30   Similar thermal resistance has been reported for *E.coli* produced Shiga Toxin.[3] While
31   pasteurization may reduce CFU's used in petri-dish or plating based safety tests, it does
32   not reduce the microbial toxins, spores or DNA encoding these toxins.
33
34   Mycotoxin monitoring in Cannabis preparations is important since aflatoxin produced by
35   Aspergillus species is a carcinogen. The clearance of aflatoxin requires the human liver
36   enzyme CYP3A4 and this liver enzyme is potently inhibited by cannabinoids[4, 5]. Modern
37   day cannabis flowers can produce up to 25% (w/v) cannabinoids presenting potent
38   inhibition of CYP3A4 and CYP2C19. Health compromised patients exposed to aflatoxin
39   and clearance-inhibiting cannabinoids raise new questions in regards to the current safety
40   tolerances to aflatoxin. Similarly, Fusarium species are known to produce fungal toxins
41   and has proven to be difficult to selectively culture with tailored media[6-8]. This is a
42   common fault of culture-based systems as carbon sources are not exclusive to certain
43   microbes and only 1% of microbial species are believed to be culturable[9].
44
45   While these risks have been well studied in the food markets, the presence of the
46   microbial populations present on cannabis flowers has never been surveyed with next
47   generation sequencing techniques [10-15]. With the publication of the Cannabis genome[16, 17]
48   and many other pathogenic microbial genomes, quantitative PCR assays have been
49   developed that can accurately quantify fungal DNA present in Cannabis samples [18]. Here,

50 we analyze the yeast and mold species present in 10 real world, dispensary-derived
51 Cannabis samples by quantitative PCR and sequencing, and demonstrate the presence of
52 several mycotoxin producing fungal strains that are not detected by widely used culture-
53 based assays.
54
55 **Results**
56 A commercially available Total Yeast and Mold qPCR assay (TYM-PathogINDICAtor,
57 Medicinal Genomics, Woburn) was used to screen for fungal DNA in a background of
58 host *Cannabis* DNA. The TYM qPCR assay targets the 18S rDNA ITS (Internal
59 Transcribed Spacer) region using modified primers described previously[19, 20]. Fungal
60 DNA amplified using these primers may also be subjected to next generation sequencing
61 to identify the contributing yeast and mold species. ITS sequencing has been widely used
62 to identify and enumerate fungal species present in a given sample[21].
63
64 We purified DNA from Cannabis samples obtained from two different geographic
65 regions (Amsterdam and Massachusetts) several years apart (2011 and 2015). The
66 majority of samples purified and screened with ITS qPCR were negative for
67 amplification signal implying reagents clean of fungal contamination. Six of the 17
68 dispensary-derived Cannabis samples tested positive for yeast and mold in the TYM
69 qPCR assay. These results were compared with the results derived from three
70 commercially available culture based detection systems for each of the 17 samples (3M
71 Petrifilm[TM] 3M Microbiology, St. Paul, MN, USA, SimPlates[TM] Biocontrol Systems,
72 Bellevue,WA, USA, BioLumix[TM] Neogen, Lansing MI, USA). Of the 6 qPCR positive
73 samples, two tested negative in all 3 culture-based assays and four tested negative in 1 or
74 2 of the culture-based assays (Table. 1). None of the qPCR negative samples tested
75 positive in any of the culture based assays. Each of the 6 discordant samples was
76 subjected to ITS sequencing to precisely identify the collection of microbes present. Four
77 additional samples from a different geographic origin (Amsterdam) were also subjected
78 to ITS sequencing, for a total of 10 Cannabis samples.
79
80 Each discordant sample presented with an array of microbial species, as shown in figure
81 2. No sample presented with a single dominant species, and each sample displayed
82 multiple species of interest. Of particular concern were the identified DNA sequences
83 from toxin producing species: *Aspergillus versicolor*[22-26], *Aspergillus terreus*[27],
84 *Penicillium citrinum*[28-30], *Penicillium paxilli*[31, 32].
85
86 We further analyzed the ITS sequence alignments using the whole genome shotgun based
87 microbiome classification software known as One Codex [33]. Nine of the ten samples
88 sequenced showed the presence of *P. paxilli* (Figure 3). To verify the accuracy of this
89 ITS phylotyping, a gene involved in the Paxilline toxin Biosynthesis pathway of *P.*
90 *paxilli* was amplified with PaxPss1 and PaxPss2 primers described by Saikia *et al*[34]. The
91 resulting 725bp amplicon (expected size) was sequenced to confirm the presence of the
92 *P. Paxilli* biosynthesis gene in the cannabis sample KD8 (Figure 4). While there are some
93 discrepancies between the two software platforms, our analysis used merged paired reads
94 with MG-RAST and correlate better with PCR results. While One Codex predicted and
95 confirmed KD8 as having the highest Paxilli content, the One Codex platform is
96 optimized for whole genome shotgun data.
97
97 With the confirmed presence of *P. paxilli*, we are curious to find out whether the toxin,
98 paxilline, is present in the samples. Development of monoclonal antibodies to paxilline

2

99   has recently been described[35], but commercial ELISA assays with sensitivity under 50ppb
100   do not appear to be available at this time. A >50ppb multiplexed ELISA assay is
101   available from Randox Food Diagostics (Crumlin,UK). Detection with LC-MS/MS has
102   also been described[36, 37], however, and experiments are underway to determine whether
103   Paxilline can be identified in the background of cannabinoids and terpenes present in
104   Cannabis samples.

105   **Discussion**
106   Several potentially harmful fungal species were detected in dispensary-derived Cannabis
107   samples by qPCR and subsequent sequencing in this study. Three different culture-based
108   assays failed to detect all of the positive samples and one, BioLumix[TM], detected only one
109   out of 7 positive samples. A review of the literature suggests that Penicillium microbes
110   can be cultured on CYA media, but some may require colder temperatures (21-24C) and
111   7 day growth times[38]. Of the Penicillium, only P. Citrinum has been previously reported
112   to culture with 3M Petri-Film[39]. In addition, several studies have demonstrated plant
113   phytochemicals and terpenoids like eugenol can inhibit the growth of fungi [40]. It is
114   possible the different water activity of the culture assay compared to the natural terpene
115   rich flower environment is contributing to the false negative test results.
116
117   Quantitative PCR is agnostic to water activity and can be performed in hours instead of
118   days. The specificity and sensitivity provides important information on samples that
119   present risks invisible to culture based systems. The draw back to qPCR is the method's
120   indifference to living or non-living DNA. While techniques exist to perform live-dead
121   qPCR, the live status of the microbes is unrelated to toxin potentially produced while the
122   microbes were alive. ELISA assays exists to screen for some toxins[41]. Current State-
123   recommended ELISA's do not detect Citrinin or Paxilline, the toxins produced by *P.*
124   *citrinum* and *P. paxilli*, respectively. The predominance of these Penicillium species in a
125   majority of the samples tested is interesting. Several Penicillium species are known to be
126   endophytes on various plant species, including *P. citrinum* [10], and this raises the question
127   of whether they are also Cannabis endophytes.
128
129   Paxilline is a tremorgenic and ataxic potassium channel blocker and has been shown to
130   attenuate the anti-seizure properties of cannabidiol in certain mouse models[42-44]. Paxilline
131   is reported to have tremorgenic effects at nanomolar concentrations and is responsible for
132   Ryegrass-staggers disease[45]. Cannabidiol is often used at micromolar concentrations for
133   seizure reduction implying sub-percentage contamination of Paxilline could still be a
134   concern. Citrinin is a mycotoxin that disrupts Ca2+ efflux in the mitochondrial
135   permeability transition pore (mPTP)[46-53]. Ryan *et al*. demonstrated that cannabidiol
136   affects this pathway suggesting a potential concern for CBD-mycotoxin interaction [54].
137   Considering the hydrophobicity of Paxilline and the recent interest in the use of
138   cannabidiol derived from cannabis flower oils for drug resistant Epilepsy, more precise
139   molecular screening of fungal toxins may be warranted [55-60].
140
141   Our survey of cannabis flowers in this study was limited. Further screening will be
142   required to define a set of tests that can adequately capture all risks. While ELISA assays
143   are easy point of use tests that can be used to detect fungal toxins, they can suffer from
144   lack of sensitivity and cross reactivity. ITS amplification and sequencing offers
145   hypothesis-free testing that can complement the lack of specificity in ELISA assays.
146   Appropriate primer design can survey a broad spectrum of microbial genomes while
147   affording rapid iteration of design. Quantitative PCR has also demonstrated single

148   molecule sensitivity and linear dynamic range over 5 orders of magnitude offering a very
149   robust approach for detection of microbial risks. This may be important for the detection
150   of nanomolar potency mycotoxins. Further studies are required to validate better
151   detection methods for these toxins and verify whether Paxilline or Citrinin are present on
152   cannabis at concentrations that present a clinical risk.
153

154   **Conclusions**
155   These results demonstrate that culture based techniques superimposed from the food
156   industry should be re-evaluated based on the known microbiome of actual Cannabis
157   flowers in circulation at dispensaries. Several mycotoxin producing molds were detected
158   that can potentially interfere with the medical use of cannabidiol. These microbes failed
159   to grow on traditional culture based platforms but were rapidly detected with molecular
160   based techniques. Further studies are required to quantitate the presence and
161   concentration of mycotoxin production.
162

163   **Methods**
164   *Culture based methods*
165   3.55ml of Tryptic Soy Broth (TSB) was used to wet 250mg of homogenized flower in a
166   whirlpack bag. TSB was aspirated from the reverse side of the 100um mesh filter and
167   placed into a Biolumix ^{TM} growth vial and spread onto a 3M Petri Film ^{TM} and a SimPlate
168   ^{TM} according to the respective manufacturers recommendations. Biolumix ^{TM} vials were
169   grown and monitored for 48 hours while Petri-films ^{TM} and SimPlates ^{TM} were grown for
170   5 days. Petri-films ^{TM} and SimPlates ^{TM} were colony counted manually by three
171   independent observers. Samples were tested on Total Coliform, Total Entero, Total
172   Aerobic, and Total Yeast and Mold. Only Total Yeast and Mold discrepancies were
173   graduated to sequencing.
174

175   *DNA Purification*
176   Plant DNA was extracted with SenSATIVAx according to manufacturers instructions
177   (Medicinal Genomics part #420001). DNA is eluted with 50ul ddH20.
178

179   *Primers used for PCR and sequencing*
180   PCR was performed using 5ul of DNA (3ng/ul) 12.5ul 2X LongAmp (NEB) with 1.25ul
181   of each 10uM MGC-ITS3 and MGC-ITS3 primer (MGC-ITS3;
182   TACACGACGTTGTAAAACGACGCATCGATGAAGAACGCAGC) and (MGC-
183   ITS3R; AGGATAACAATTTCACACAGGATTTGAGCTCTTGCCGCTTCA) with
184   10ul ddH20 for a 25ul total reaction. An initial 95C 5 minute denaturization was
185   performed followed by 40 cycles of 95C for 15s and 65C for 90s. Samples were purified
186   with 75ul SenSATIVAx, washed twice with 100ul 70% EtOH and bench dried for
187   5minutes at room temperature. Samples were eluted in 25ul ddH20.
188

189   *Tailed PCR Cloning and Sequencing*
190   DNA libraries were constructed with 250ng DNA using NEB's NEBNext Quick
191   ligation module (NEB # E6056S). End Repair used 3ul of Enzyme Mix, 6.5ul of
192   Reagent Mix, 55.5ul of DNA + ddH20. Reaction was incubated at 30C for 20 minutes.
193   After End Repair, Ligation was performed directly with 15ul of Blunt End TA Mix,
194   2.5ul of Ilumina Adaptor (10uM) and 1ul of Ligation enhancer (assumed to be 20%
195   PEG 6000). After 15-minute ligation at 25C, 3ul of USER enzyme was added to digest
196   the hairpin adaptors and prepare for PCR. The USER enzyme was tip-mixed and

197 incubated at 37C for 20 minutes. After USER digestion, 86.5ul of SenSATIVAx was
198 added and mixed. The samples were placed on a magnet for 15 minutes until the
199 beads cleared and the supernatant could be removed. Beads were washed twice
200 with 150ul of 70% EtOH. Beads were left for 10 minute to air dry and then eluted in
201 25ul of 10mM Tris-HCl.
202
203 *Library PCR*
204 25ul 2X Q5 Polymerase was added to 23ul of DNA with 1ul of i7 index primer (25uM)
205 and 1ul Universal primer (25um). After an initial 95C for 10 secs, the library was
206 amplified for 15 cycles of 95C 10sec, 65C 90sec. Samples were purified by mixing 75ul
207 of SenSATIVAx into the PCR reaction. The samples were placed on a magnet for 15
208 minutes until the beads cleared and the supernatant could be removed. Beads were
209 washed twice with 150ul of 70% EtOH. Beads were left for 10 minute to air dry and
210 then eluted in 25ul of 10mM Tris-HCl. Samples were prepared for sequencing on the
211 MiSeq V2 chemistry according to the manufactures instructions. 2x250bp reads
212 were selected to obtain maximal ITS sequence information.
213
214 *PaxP Verification PCR*
215 Primers described by Shirazi-zand et al. were utilized to amplify a segment of the 725bp
216 PaxP gene. 25ul LongAmp (NEB) 4ul 10uM Primer, 1ul DNA (14ng/ul), 20ul ddH20 to
217 make a 50ul PCR reaction. Cycling conditions we slightly modified to accommodate a
218 different polymerase. 95C for 30s followed by 28 cycles of 95C 15s, 55C for 30sec, 65C
219 2.5 minutes. Samples were purified with 50ul of SenSATIVAx as described above. 1ul of
220 purified PCR product was sized on Agilent HS 2000 chip. Nextera libraries and
221 sequencing were performed according to instructions from Illumina using 2x75bp
222 sequencing on a version 2 MiSeq.
223
224 *Analysis*
225 Reads were demultiplex and trimmed with Casava 1.8.2 and trim_galore. FLASH [61] was
226 used to merge the reads using max_overlap 150. The reads were aligned to microbial
227 references using MG-RAST[62]. Alignments and classifications were confirmed with a
228 second software tool from One Codex and critical pathways identified for further
229 evaluation with PCR of toxin producing genes. Reads are deposited in NCBI under SRA
230 accession: SRP065410. Nextera 2x75bp sequencing of the PaxP gene was mapped to
231 accession number HM171111.1 with CLCbio Workstation V4 at 98% identity over
232 80% of the read. One Codex analysis was put into Public mode under the following
233 public URLs:
234
235 Australian Bastard:
236 https://app.onecodex.com/analysis/public/201e7f1642e04a3c
237 https://app.onecodex.com/analysis/public/58f1e03c10434bfa
238
239 KD4:
240 https://app.onecodex.com/analysis/public/2e86e262817246c4
241 https://app.onecodex.com/analysis/public/1abd5b60446140a0
242
243 KD6:
244 https://app.onecodex.com/analysis/public/a92d3dff5485499d
245 https://app.onecodex.com/analysis/public/8d72e2514e564ecd

5

246
247 KD8:
248 https://app.onecodex.com/analysis/public/8d72e2514e564ecd
249 https://app.onecodex.com/analysis/public/d6e2e0bcfba3469f
250
251 Liberty Haze:
252 https://app.onecodex.com/analysis/public/7bcd650fa5544f2c
253 https://app.onecodex.com/analysis/public/7f0feb6cb0a94d56
254
255 Girls Scout Cookie:
256 https://app.onecodex.com/analysis/public/a71b1ce8331c461d
257 https://app.onecodex.com/analysis/public/8d6f10c7ee684f93
258 Jakes Grape:
259 https://app.onecodex.com/analysis/public/bc8af5ed19e5407a
260 https://app.onecodex.com/analysis/public/99d7a4a2f7af486b
261 RECON:
262 https://app.onecodex.com/analysis/public/8a22a16cc2e24731
263 https://app.onecodex.com/analysis/public/0af6ae26a01f48d5
264
265 GreenCrack:
266 https://app.onecodex.com/analysis/public/6114843d2eb3425e
267 https://app.onecodex.com/analysis/public/3eee642786c54a88
268
269 LA Confidential:
270 https://app.onecodex.com/analysis/public/01e8aefb0d4f4f62
271 https://app.onecodex.com/analysis/public/b74c2988fcd84e38
272
273 NYC Diesel:
274 https://app.onecodex.com/analysis/public/441cfad759f64dcc
275 https://app.onecodex.com/analysis/public/d97b39cae96c4a44
276
277 Author Contributions:
278 KJM designed the study and performed the One-Codex analysis
279 JS designed and ran the culture and qPCR laboratory experiments
280 LZ assisted in the figure generation and laboratory experiment
281 YH assisted in sequencing and PCR confirmation of Pax.
282 VT- read alignment, MG-RAST, primer design and analysis.
283 TF- Sample tracking software, figure generations, ITS software comparisons
284 DS- Manuscript construction and review
285

288
289 1.   Fujikawa, H. & Itoh, T. Tailing of thermal inactivation curve of Aspergillus
290   niger spores. *Applied and environmental microbiology* **62**, 3745-3749 (1996).
291 2.   Kabak, B. & Dobson, A.D. Biological strategies to counteract the effects of
292   mycotoxins. *Journal of food protection* **72**, 2006-2016 (2009).

293 3.   Rasooly, R. & Do, P.M. Shiga toxin Stx2 is heat-stable and not inactivated by
294   pasteurization. *International journal of food microbiology* **136**, 290-294
295   (2010).
296 4.   Langouet, S. et al. Metabolism of aflatoxin B1 by human hepatocytes in
297   primary culture. *Adv Exp Med Biol* **387**, 439-442 (1996).
298 5.   Yamaori, S., Ebisawa, J., Okushima, Y., Yamamoto, I. & Watanabe, K. Potent
299   inhibition of human cytochrome P450 3A isoforms by cannabidiol: role of
300   phenolic hydroxyl groups in the resorcinol moiety. *Life Sci* **88**, 730-736
301   (2011).
302 6.   Bragulat, M.R., Martinez, E., Castella, G. & Cabanes, F.J. Selective efficacy of
303   culture media recommended for isolation and enumeration of Fusarium spp.
304   *Journal of food protection* **67**, 207-211 (2004).
305 7.   Castella, G., Bragulat, M.R., Rubiales, M.V. & Cabanes, F.J. Malachite green
306   agar, a new selective medium for Fusarium spp. *Mycopathologia* **137**, 173-
307   178 (1997).
308 8.   Desjardins, A.E. & Proctor, R.H. Molecular biology of Fusarium mycotoxins.
309   *International journal of food microbiology* **119**, 47-50 (2007).
310 9.   Stewart, E.J. Growing unculturable bacteria. *Journal of bacteriology* **194**,
311   4151-4160 (2012).
312 10. Kusari P, K.S., Spiteller M, Kayser O Endophytic fungi harbored in Cannabis
313   sativa L.: diversity and potential as biocontrol agents against host
314   plantspecific phytopathogens. *Fungal Diversity.* **60**, 137–151 (2013).
315 11. McPartland Fungal pathogens of Cannabis sativa in Illinois. *Phytopathology*
316   **72:797** (1983).
317 12. McPartland Common names for diseases of Cannabis sativa L. . *Plant Dis* **75**,
318   226–227 (1991).
319 13. McPartland Microbiological contaminants of marijuana. *J. Int Hemp Assoc* **1**,
320   41–44 (1994).
321 14. McPartland Cannabis pathogens X: Phoma, Ascochyta and Didymella species.
322   *Mycologia* **86**, 870–878 (1995).
323 15. McPartland A review of Cannabis diseases. . *J Int Hemp Assoc* **3**, 19–23
324   (1996).
325 16. van Bakel, H. et al. The draft genome and transcriptome of Cannabis sativa.
326   *Genome biology* **12**, R102 (2011).
327 17. McKernan The Cannabis Sativa Genome [Online] Available at:
328   (https://aws.amazon.com/datasets/the-cannabis-sativa-genome/) Accessed
329   on 13 September 2015. (2011).
330 18. McKernan *[Online] Available at:*
331   *http://www.icrs.co/SYMPOSIUM.2015/ICRS2015.Preliminary.Programme.pdf,*
332   *Accessed on 20 October 2015* (2015).
333 19. Borneman, J. & Hartin, R.J. PCR primers that amplify fungal rRNA genes from
334   environmental samples. *Applied and environmental microbiology* **66**, 4356-
335   4360 (2000).
336 20. White, T.J., T. Bruns, S. Lee & J. Taylor. Amplification and direct sequencing of
337   fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to*
338   *Methods and Applications*, 315-322 (1990).

339   21.   Mason, M., Cheung, I. & Walker, L. Creating a geospatial database of risks and
340   resources to explore urban adolescent substance use. *Journal of prevention &*
341   *intervention in the community* **37**, 21-34 (2009).
342   22.   Abd Alla, E.A., Metwally, M.M., Mehriz, A.M. & Abu Sree, Y.H. Sterigmatocystin:
343   incidence, fate and production by Aspergillus versicolor in Ras cheese. *Die*
344   *Nahrung* **40**, 310-313 (1996).
345   23.   Aly, S.A. & Elewa, N.A. The effect of Egyptian honeybee propolis on the
346   growth of Aspergillus versicolor and sterigmatocystin biosynthesis in Ras
347   cheese. *The Journal of dairy research* **74**, 74-78 (2007).
348   24.   Engelhart, S. et al. Occurrence of toxigenic Aspergillus versicolor isolates and
349   sterigmatocystin in carpet dust from damp indoor environments. *Applied and*
350   *environmental microbiology* **68**, 3886-3890 (2002).
351   25.   Kocic-Tanackov, S. et al. Effects of onion (Allium cepa L.) and garlic (Allium
352   sativum L.) essential oils on the Aspergillus versicolor growth and
353   sterigmatocystin production. *Journal of food science* **77**, M278-284 (2012).
354   26.   Song, F. et al. Three new sterigmatocystin analogues from marine-derived
355   fungus Aspergillus versicolor MF359. *Applied microbiology and biotechnology*
356   **98**, 3753-3758 (2014).
357   27.   El-Sayed Abdalla, A., Zeinab Kheiralla, M., Sahab, A. & Hathout, A. Aspergillus
358   terreus and its toxic metabolites as a food contaminant in some Egyptian
359   Bakery products and grains. *Mycotoxin research* **14**, 83-91 (1998).
360   28.   Ames, D.D., Wyatt, R.D., Marks, H.L. & Washburn, K.W. Effect of citrinin, a
361   mycotoxin produced by Penicillium citrinum, on laying hens and young
362   broiler chicks. *Poultry science* **55**, 1294-1301 (1976).
363   29.   Mazumder, P.M., Mazumder, R., Mazumder, A. & Sasmal, D.S. Antimicrobial
364   activity of the mycotoxin citrinin obtained from the fungus penicillium
365   citrinum. *Ancient science of life* **21**, 191-197 (2002).
366   30.   Park, S.Y. et al. Citrinin, a mycotoxin from Penicillium citrinum, plays a role in
367   inducing motility of Paenibacillus polymyxa. *FEMS microbiology ecology* **65**,
368   229-237 (2008).
369   31.   Itoh, Y., Johnson, R. & Scott, B. Integrative transformation of the mycotoxin-
370   producing fungus, Penicillium paxilli. *Current genetics* **25**, 508-513 (1994).
371   32.   Shibayama, M., Ooi, K., Johnson, R., Scott, B. & Itoh, Y. Suppression of tandem-
372   multimer formation during genetic transformation of the mycotoxin-
373   producing fungus Penicillium paxilli by disrupting an orthologue of
374   Aspergillus nidulans uvsC. *Current genetics* **42**, 59-65 (2002).
375   33.   Minot, S.S., Krumm, N. & Greenfield, N.B. One Codex: A Sensitive and Accurate
376   Data Platform for Genomic Microbial Identification. *bioRxiv* (2015).
377   34.   Saikia, S., Parker, E.J., Koulman, A. & Scott, B. Defining paxilline biosynthesis
378   in Penicillium paxilli: functional characterization of two cytochrome P450
379   monooxygenases. *The Journal of biological chemistry* **282**, 16829-16837
380   (2007).
381   35.   Maragos, C.M. Development and Evaluation of Monoclonal Antibodies for
382   Paxilline. *Toxins* **7**, 3903-3915 (2015).
383   36.   Vishwanath, V., Sulyok, M., Labuda, R., Bicker, W. & Krska, R. Simultaneous
384   determination of 186 fungal and bacterial metabolites in indoor matrices by
385   liquid chromatography/tandem mass spectrometry. *Analytical and*
386   *bioanalytical chemistry* **395**, 1355-1372 (2009).

387 37.   Uhlig, S., Egge-Jacobsen, W., Vralstad, T. & Miles, C.O. Indole-diterpenoid
388   profiles of Claviceps paspali and Claviceps purpurea from high-resolution
389   Fourier transform Orbitrap mass spectrometry. *Rapid communications in*
390   *mass spectrometry : RCM* **28**, 1621-1634 (2014).
391 38.   Houbraken, J., Frisvad, J.C. & Samson, R.A. Taxonomy of Penicillium section
392   Citrina. *Studies in mycology* **70**, 53-138 (2011).
393 39.   3M http://multimedia.3m.com/mws/media/898592O/3m-petrifilm-rapid-
394   yeast-mold-count-plate.pdf.
395 40.   Zare, M., Shams-Ghahfarokhi, M., Ranjbar-Bahadori, S., Allameh, A. &
396   Razzaghi-Abyaneh, M. Comparative study of the major Iranian cereal
397   cultivars and some selected spices in relation to support Aspergillus
398   parasiticus growth and aflatoxin production. *Iranian biomedical journal* **12**,
399   229-236 (2008).
400 41.   Labs, R. [Online] Available at:
401   http://www.romerlabs.com/en/knowledge/mycotoxins/, Accessed on Oct,
402   2015.
403 42.   Shirazi-zand, Z., Ahmad-Molaei, L., Motamedi, F. & Naderi, N. The role of
404   potassium BK channels in anticonvulsant effect of cannabidiol in
405   pentylenetetrazole and maximal electroshock models of seizure in mice.
406   *Epilepsy & behavior : E&B* **28**, 1-7 (2013).
407 43.   Sabater-Vilar, M., Nijmeijer, S. & Fink-Gremmels, J. Genotoxicity assessment
408   of five tremorgenic mycotoxins (fumitremorgen B, paxilline, penitrem A,
409   verruculogen, and verrucosidin) produced by molds isolated from fermented
410   meats. *Journal of food protection* **66**, 2123-2129 (2003).
411 44.   Sanchez-Pastor, E. et al. Cannabinoid receptor type 1 activation by
412   arachidonylcyclopropylamide in rat aortic rings causes vasorelaxation
413   involving calcium-activated potassium channel subunit alpha-1 and calcium
414   channel, voltage-dependent, L type, alpha 1C subunit. *European journal of*
415   *pharmacology* **729**, 100-106 (2014).
416 45.   Imlach, W.L. et al. The molecular mechanism of "ryegrass staggers," a
417   neurological disorder of K+ channels. *The Journal of pharmacology and*
418   *experimental therapeutics* **327**, 657-664 (2008).
419 46.   Chagas, G.M., Campello, A.P. & Kluppel, M.L. Mechanism of citrinin-induced
420   dysfunction of mitochondria. I. Effects on respiration, enzyme activities and
421   membrane potential of renal cortical mitochondria. *Journal of applied*
422   *toxicology : JAT* **12**, 123-129 (1992).
423 47.   Chagas, G.M., Oliveira, B.M., Campello, A.P. & Kluppel, M.L. Mechanism of
424   citrinin-induced dysfunction of mitochondria. II. Effect on respiration,
425   enzyme activities, and membrane potential of liver mitochondria. *Cell*
426   *biochemistry and function* **10**, 209-216 (1992).
427 48.   Chagas, G.M., Oliveira, M.A., Campello, A.P. & Kluppel, M.L. Mechanism of
428   citrinin-induced dysfunction of mitochondria. IV--Effect on Ca2+ transport.
429   *Cell biochemistry and function* **13**, 53-59 (1995).
430 49.   Chagas, G.M., Oliveira, M.B., Campello, A.P. & Kluppel, M.L. Mechanism of
431   citrinin-induced dysfunction of mitochondria. III. Effects on renal cortical and
432   liver mitochondrial swelling. *Journal of applied toxicology : JAT* **15**, 91-95
433   (1995).

434 50. Da Lozzo, E.J., Oliveira, M.B. & Carnieri, E.G. Citrinin-induced mitochondrial
435   permeability transition. *Journal of biochemical and molecular toxicology* **12**,
436   291-297 (1998).
437 51. Jeswal, P. Citrinin-induced chromosomal abnormalities in the bone marrow
438   cells of Mus musculus. *Cytobios* **86**, 29-33 (1996).
439 52. Ribeiro, S.M., Campello, A.P., Chagas, G.M. & Kluppel, M.L. Mechanism of
440   citrinin-induced dysfunction of mitochondria. VI. Effect on iron-induced lipid
441   peroxidation of rat liver mitochondria and microsomes. *Cell biochemistry and*
442   *function* **16**, 15-20 (1998).
443 53. Ribeiro, S.M., Chagas, G.M., Campello, A.P. & Kluppel, M.L. Mechanism of
444   citrinin-induced dysfunction of mitochondria. V. Effect on the homeostasis of
445   the reactive oxygen species. *Cell biochemistry and function* **15**, 203-209
446   (1997).
447 54. Ryan, D., Drysdale, A.J., Lafourcade, C., Pertwee, R.G. & Platt, B. Cannabidiol
448   targets mitochondria to regulate intracellular Ca2+ levels. *The Journal of*
449   *neuroscience : the official journal of the Society for Neuroscience* **29**, 2053-
450   2063 (2009).
451 55. Devinsky, O., Whalley, B.J. & Di Marzo, V. Erratum to: Cannabinoids in the
452   Treatment of Neurological Disorders. *Neurotherapeutics : the journal of the*
453   *American Society for Experimental NeuroTherapeutics* **12**, 910 (2015).
454 56. Devinsky, O., Whalley, B.J. & Di Marzo, V. Cannabinoids in the Treatment of
455   Neurological Disorders. *Neurotherapeutics : the journal of the American*
456   *Society for Experimental NeuroTherapeutics* **12**, 689-691 (2015).
457 57. Friedman, D. & Devinsky, O. Cannabinoids in the Treatment of Epilepsy. *The*
458   *New England journal of medicine* **373**, 1048-1058 (2015).
459 58. Rosenberg, E.C., Tsien, R.W., Whalley, B.J. & Devinsky, O. Cannabinoids and
460   Epilepsy. *Neurotherapeutics : the journal of the American Society for*
461   *Experimental NeuroTherapeutics* **12**, 747-768 (2015).
462 59. Cilio, M.R., Thiele, E.A. & Devinsky, O. The case for assessing cannabidiol in
463   epilepsy. *Epilepsia* **55**, 787-790 (2014).
464 60. Devinsky, O. et al. Cannabidiol: pharmacology and potential therapeutic role
465   in epilepsy and other neuropsychiatric disorders. *Epilepsia* **55**, 791-802
466   (2014).
467 61. Magoc, T. & Salzberg, S.L. FLASH: fast length adjustment of short reads to
468   improve genome assemblies. *Bioinformatics* **27**, 2957-2963 (2011).
469 62. Glass, E.M., Wilkening, J., Wilke, A., Antonopoulos, D. & Meyer, F. Using the
470   metagenomics RAST server (MG-RAST) for analyzing shotgun metagenomes.
471   *Cold Spring Harbor protocols* **2010**, pdb prot5368 (2010).
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**Figures: Cannabis microbiome sequencing reveals mycotoxic fungi native to dispensary grade cannabis flowers**

Total Yeast & Mold
(10,000 CFU/g)

Samples	Yeast/Mold			
	Simplate®(CFU/g)	3M™ (CFU/g)	Biolumix	Cq
KD4	0	0	pass	21.71 (f)
KD8	0	0	pass	22.5 (f)
PC3	0	0	pass	>40 (p)
White Widow	0	0	pass	>40 (p)
KD1	0	0	pass	29.33 (p)
KD2	0	0	pass	>40 (p)
KD3	0	0	pass	30.16 (p)
KD5	1000 (p)	6000 (p)	pass	27.76 (p)
KD6	3000 (p)	19000 (f)	pass	24.72 (f)
KD7	0	0	pass	>40 (p)
Liberty Haze	172000 (f)	89000 (f)	pass	24.02 (f)
Blueberry Kush	0	0	pass	37.99 (p)
Blueberry Kush (Experimental)	>738000 (f)	too high (f)	fail	15.71 (f)
Girl Scout Cookies	>738000 (f)	Too high (f)	pass	19.66 (f)
Jake's Grape	>738000 (f)	Too high (f)	pass	24.56 (f)
Serious Happiness	0	0	pass	>40 (p)
White Rhino	0	3000 (p)	pass	>40 (p)

Table 1. Samples were cultured with 3 different techniques and compared to quantitative PCR (qPCR). Biolumix had the lower sensitivity failing to pick up 4/17 samples detected with other culture-based platforms. qPCR identified 2 samples that were not picked by any other method. Positive qPCR samples were sequenced to identify the contributing signal. Highlighted samples fail the 10,000 CFU/g cutoffs which equates to a Cq of 26 on the qPCR assay according to the manufacturers instructions. (f) is Fail or over 10,000 CFU/g. (p) is Pass or under 10,000 CFU/g.
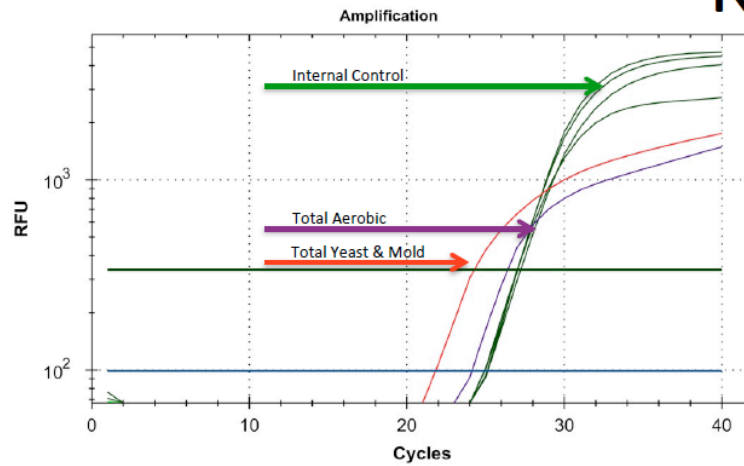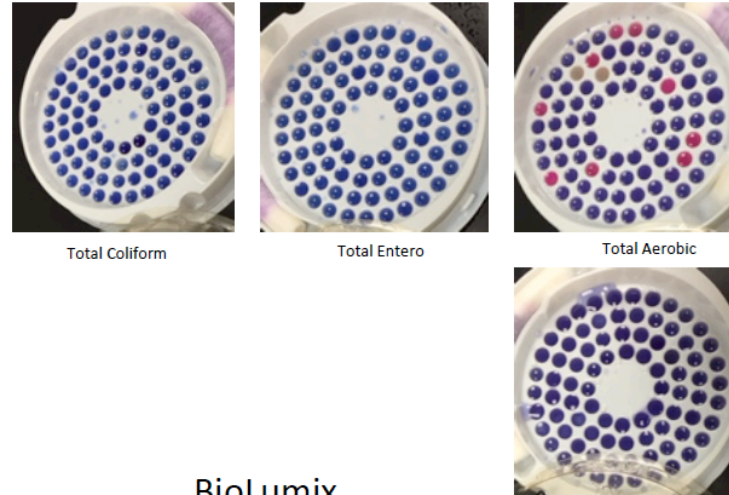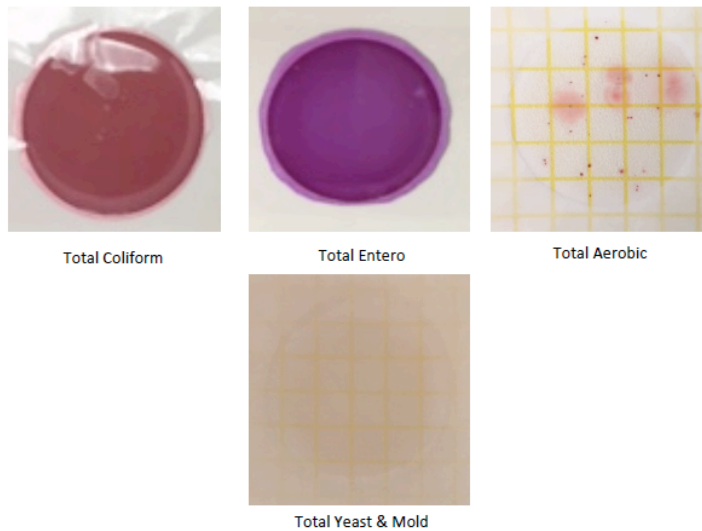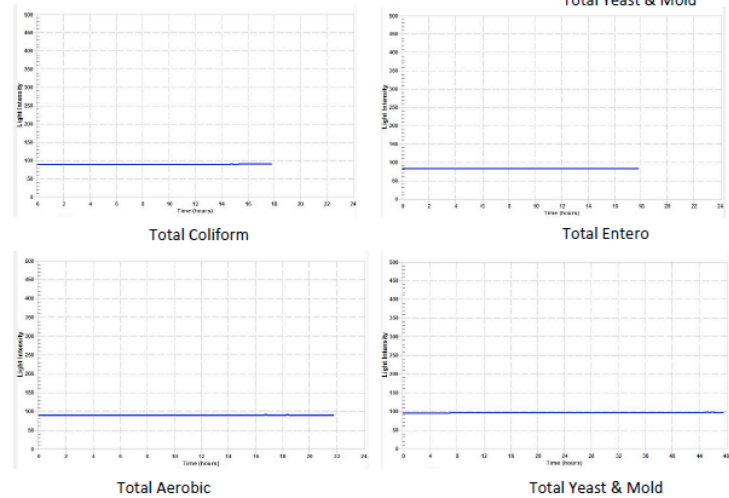
Figure 1A. qPCR signal from TYM (red line) test run concurrently (multiplexed) with a plant internal control marker (green line). This marker targets a conserved region in the cannabis genome and should show up in every assay (Upper left). SimPlates count the number of discolored wells (Purple to pink) as a proxy for CFU/gram. Only total aerobic show growth (Upper Right). Petrifilm only demonstrate colonies on Total Aerobic platings (lower left). Biolumix demonstrate no signal across all 4 test (lower right).
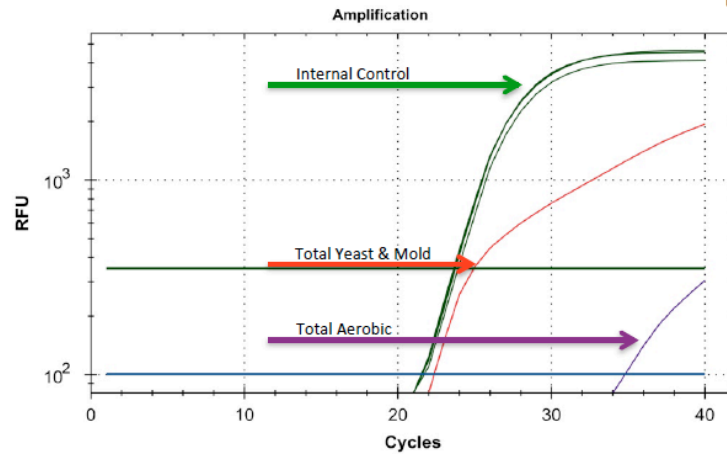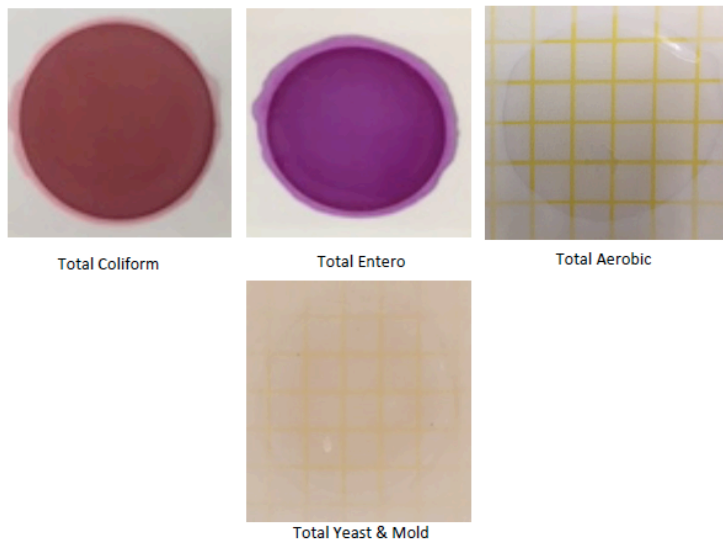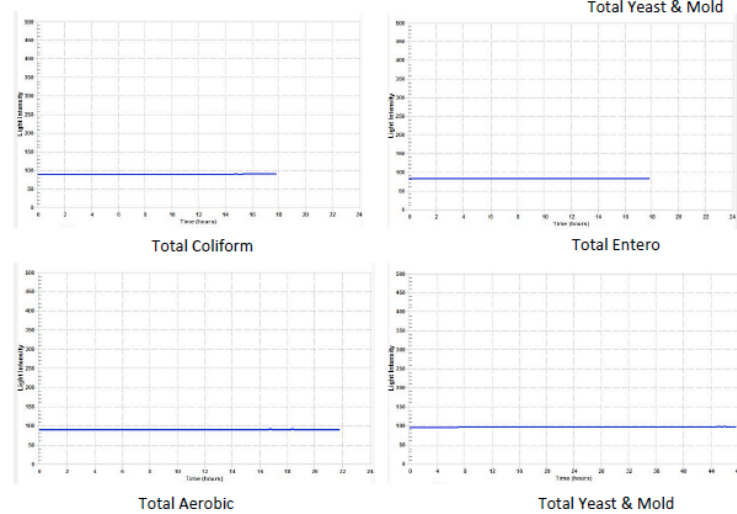
Figure 1B. Sample KD8 fails to culture any Total Yeast and mold yet demonstrates significant TYM qPCR signal. Sample was graduated to ITS based next generation sequencing.
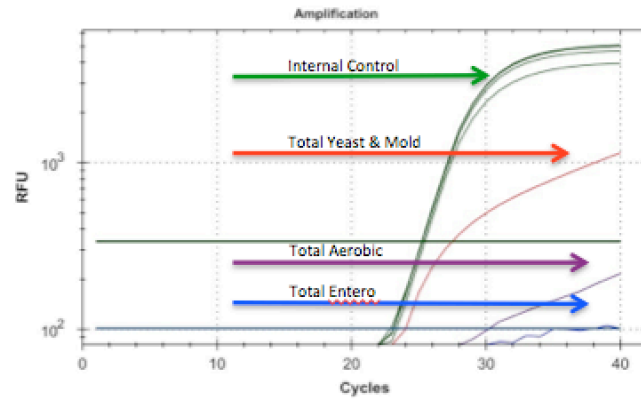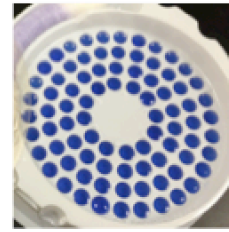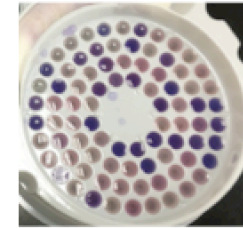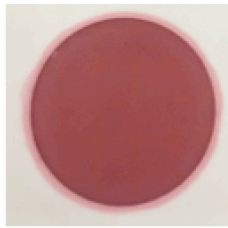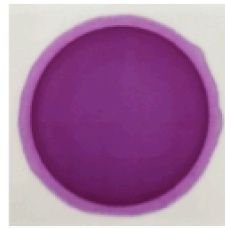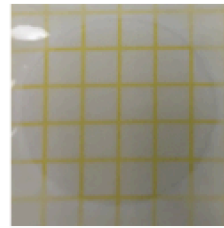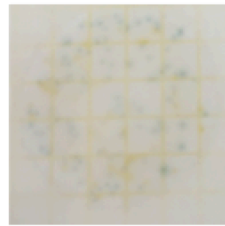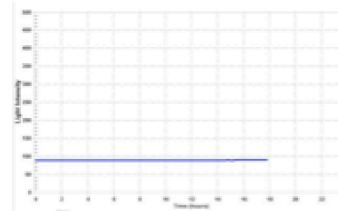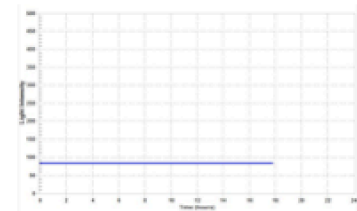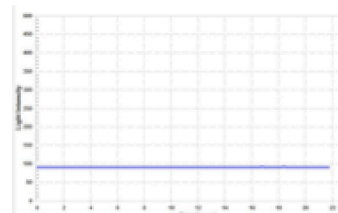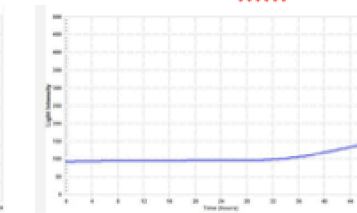
Figure 1C. Sample Liberty Haze was tested with 3 culture based methods and compared to qPCR. Sample was graduated to ITS based next generation sequencing.
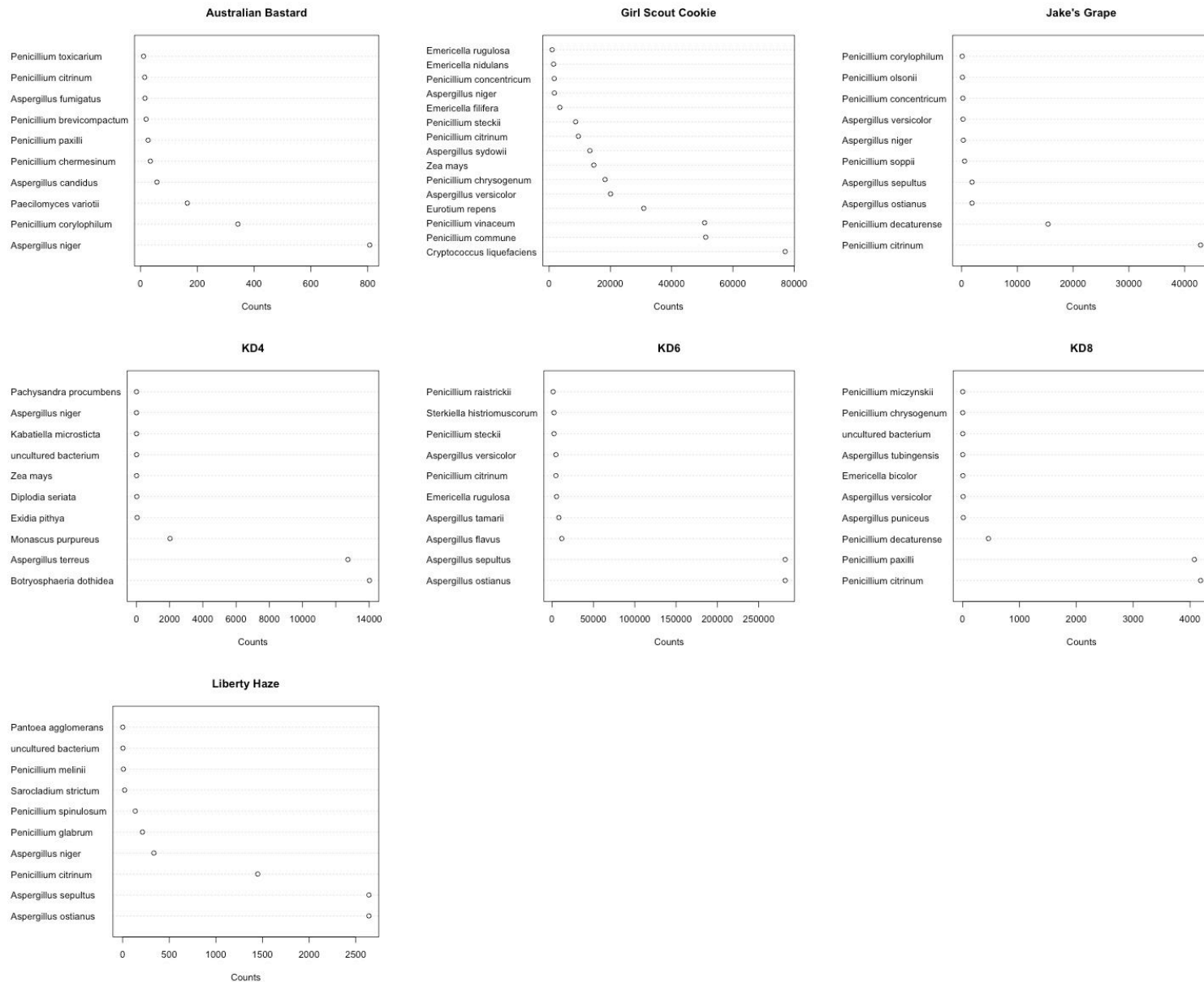
Figure 2- DNA sequencing of ITS3 amplicons from culture negative samples that are qPCR positive for Total Yeast and Mold tests. Penicillium and Aspergillus are commonly found (Y axis) but at different read counts in each sample (X axis). Read counts are more a reflection of sample normalization for sequencing than inter sample quantitation provided by qPCR.
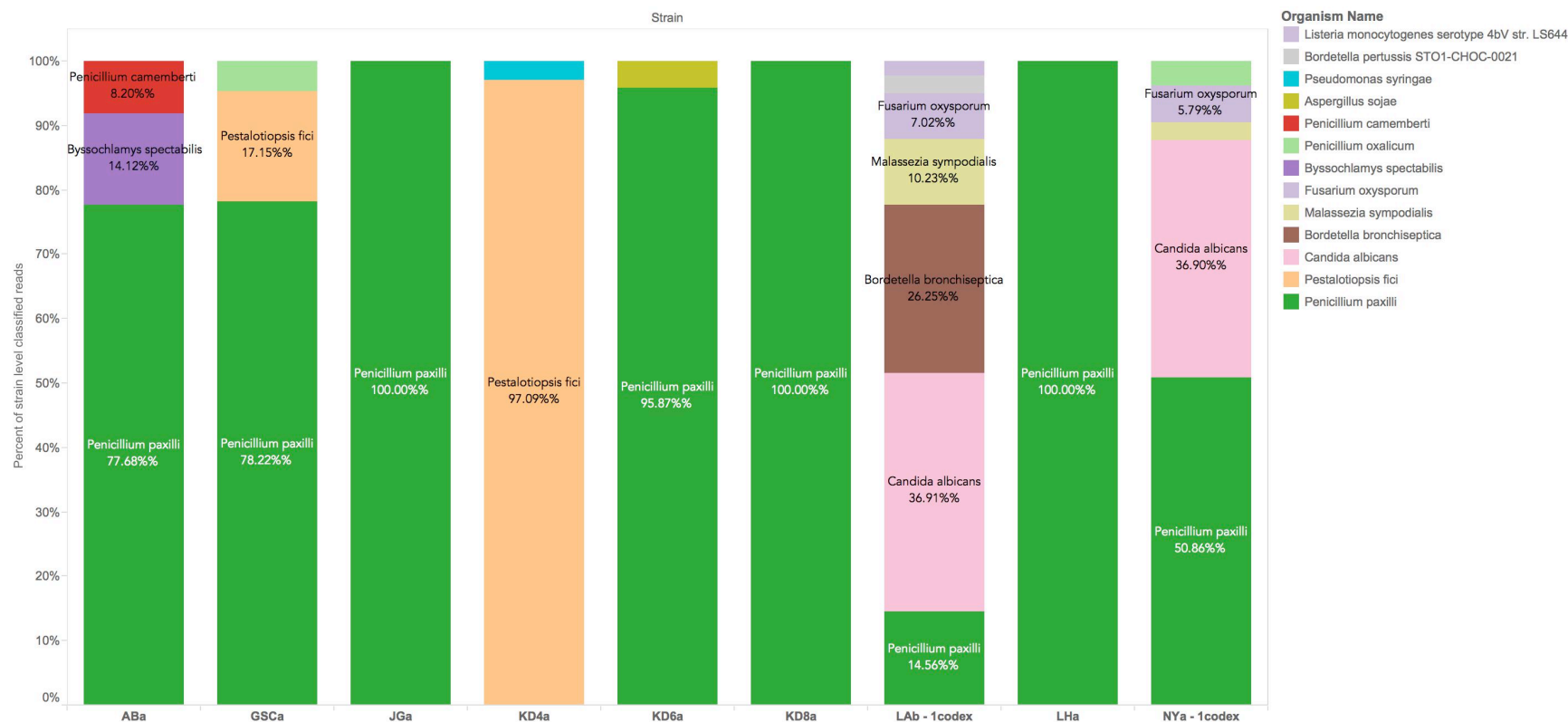
Figure 3- One Codex classification of ITS reads. P.Paxilli is the most frequently found contaminant in Cannabis flowers. P.Citrinum is not in the One Codex database at this time. One Codex utilizes a fast k-mer based approach for whole genome shotgun classification and can be influenced by read trimming and database content. The reads provided to MG-RAST were trimmed and FLASH'd (Paired end reads merged when overlapping) prior to classification. K-mer based approaches can significantly differ from longer word size methods and this underscores the importance of confirmatory PCR in microbiome analysis.

Figure 4. PaxP PCR demonstrates amplification of a 725bp band in sample KD8(A). PCR products were made into a shotgun library with Nextera and sequenced on an Illumina MiSeq with 2x75bp reads to over 10,000X coverage. Reads were mapped with CLCbio 4 to NCBI Accession number HM171111.1. Paired reads are displayed as blue lines, Green and Red lines are unpaired reads. Read Coverage over the amplicon is depicted in a blue histogram over the cluster while paired end read distance is measured in a red histogram over the region. Off target read mapping is limited (B). Alignment of PCR primers to P.Paxilli reference shows a 5 prime mismatch that is a result of the primers being designed to target spliced RNA according to Saikia *et al*.