RESEARCH ARTICLE

REVISED *Cannabis* microbiome sequencing reveals several mycotoxic fungi native to dispensary grade *Cannabis* flowers [version 2; referees: 2 approved]

Kevin McKernan, Jessica Spangler, Lei Zhang, Vasisht Tadigotla, Yvonne Helbert, Theodore Foss, Douglas R. Smith

Medicinal Genomics Corporation, Woburn, MA, USA

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Abstract

The Center for Disease Control estimates 128,000 people in the U.S. are hospitalized annually due to food borne illnesses. This has created a demand for food safety testing targeting the detection of pathogenic mold and bacteria on agricultural products. This risk extends to medical *Cannabis* and is of particular concern with inhaled, vaporized and even concentrated *Cannabis* products . As a result, third party microbial testing has become a regulatory requirement in the medical and recreational *Cannabis* markets, yet knowledge of the *Cannabis* microbiome is limited. Here we describe the first next generation sequencing survey of the fungal communities found in dispensary based *Cannabis* flowers by ITS2 sequencing, and demonstrate the sensitive detection of several toxigenic *Penicillium* and *Aspergillus* species, including *P. citrinum and P. paxilli,* that were not detected by one or more culture-based methods currently in use for safety testing.

Keywords

Cannabis , Microbiome , Mycotoxins , Cannabidiol , Paxilline , Citrinin , qPCR , Culture , Next generation sequencing

Open Peer Review

Referee Status: ✓ ✓

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1 **John McPartland**, GW Pharmaceutical, UK

2 **Donald Cooper**, Mobile Assay Inc., USA

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Corresponding author: Kevin McKernan (kevin.mckernan@courtagen.com)

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REVISED **Amendments from Version 1**

The statement in the abstract saying that the findings "demonstrate the limitations in the culture-based regulations" was removed. We added a brief mention of the limited literature relating to Cannabis microbiomes to the introduction. Clarification as to the scope of the study including only analysis of fungal microbiomes was added. We revised paragraph 2 of the introduction to focus on the numerous literature reports of pulmonary aspergillosis associated with cannabis use instead of potential dangers of mycotoxin toxicity. We provided additional background on the overlap of cannabinoid and mycotoxin metabolism via cytochrome P450 system. Figure 2 was revised and the figure legend expanded. We removed spurious hits to non-fungal species and reported only high-confidence species detected with 10 reads or more. This resulted in a higher number of species reported for some samples and fewer for others. The first paragraph of the discussion section was expanded to describe the additional findings. We added a sentence to the end of the second paragraph of the discussion mentioning the two existing publications on endophytic fungi in Cannabis. We removed the comment on potential growth inhibition relating to terpenoids. With respect to potential paxilline contamination, we de-emphasized the concern based solely on the detection of *P. paxilli*, and stated instead that if the results were verified by tests indicating high levels of paxilline then it may be a cause for concern. The comments relating to the sensitivity of ELISA assays were deleted. Some clarification was added to the concluding paragraph to emphasize the need to ensure that all species of potential concern can be detected, and also the need for additional studies to characterize a broader diversity samples, including measurements of toxin levels where relevant.

See referee reports

Introduction

Our knowledge of the natural microbiome of field-grown *Cannabis* in terms of rhizosphere bacteria, and endophytic fungi is limited to just a few focused studies[1–3]. Very little is known about the potential for bacterial and fungal contamination on medicinal *Cannabis*. Nevertheless, many states in the U.S. are now crafting regulations for detection of microbial contamination on *Cannabis* in the absence of any comprehensive survey of actual samples. A few of these regulations are inducing growers to "heat kill" or pasteurize *Cannabis* flowers to lower microbial content. While this seems a harmless suggestion, we must remain aware of how these drying techniques may create false negatives in culture-based safety tests used to monitor colony-forming units (CFU). Even though pasteurization may be effective at sterilizing some of the microbial content, it does not eliminate various pathogenic toxins or spores. *Aspergillus* spores and mycotoxins are known to resist pasteurization[4,5]. Similar thermal resistance has been reported for *E. coli* produced Shiga toxin[6]. While pasteurization may reduce CFU's used in petri-dish or plating based safety tests, it does not reduce the microbial toxins, spores or DNA encoding these toxins.

Monitoring for mycotoxic fungi in cannabis preparations has been recommended as part of routine safety testing by the Cannabis Safety Institute. A major driver for this recommendation has been numerous reported cases of serious or fatal pulmonary Aspergillosis associated with marijuana smoking in immunocompromised patients[7–9]. The major cannabinoids have been shown to be potent inhibitors of several cytochrome P450 enzymes at therapeutic concentrations, including 1A1, 1A2, 1B1 2B6, 2C19, 2D6, 3A4 and 3A5[10]. Some of these enzymes have been implicated in the metabolism of the fungal toxins aflatoxin and ochratoxin[11–13]. This raises questions about potential interactions and appropriate safety tolerances for mycotoxins in patients being treated with cannabinoid therapeutics. In addition, some *Fusarium* species that produce toxins have proven to be difficult to selectively culture with tailored media[14–16]. This is a common problem associated with culture-based systems as carbon sources are not exclusive to certain microbes and only 1% of microbial species are believed to be culturable[17].

While the risks of mycotoxic fungal contamination have been well studied in the food markets, the presence of the fungal populations present on *Cannabis* flowers has never been surveyed with next generation sequencing techniques[18–23]. With the publication of the *Cannabis* genome[24,25] and many other pathogenic microbial genomes, quantitative PCR assays have been developed that can accurately quantify fungal DNA present in *Cannabis* samples[26]. Here, we analyze the yeast and mold species present in 10 real world, dispensary-derived *Cannabis* samples by quantitative PCR and sequencing, and demonstrate the presence of several mycotoxin producing fungal strains that are not detected by widely used culture-based assays.

Methods

Culture-based methods

The culture-based methods selected for testing here represent those currently in use by established medicinal *Cannabis* safety testing laboratories. 3.55ml of tryptic soy broth (TSB) was used to wet 250mg of homogenized flower in a whirlpack bag. TSB was aspirated from the reverse side of the 100µm mesh filter and placed into a Biolumix™ growth vial and spread onto a 3M Petri Film™ and a SimPlate™ (3M Petrifilm™ 3M Microbiology, St. Paul, MN, USA; SimPlates™ Biocontrol Systems, Bellevue, WA, USA; BioLumix™ Neogen, Lansing MI, USA) according to the respective manufacturers' recommendations. Biolumix™ vials were grown and monitored for 48 hours while Petri-films™ and SimPlates™ were grown for 5 days. Petri-films™ and SimPlates™ were colony counted manually by three independent observers. Samples were tested on total coliform, total entero, total aerobic, and total yeast and mold. Only total yeast and mold discrepancies were graduated to sequencing.

DNA purification

Plant DNA was extracted with SenSATIVAx according to manufacturers' instructions (Medicinal Genomics part #420001). DNA was eluted with 50µl ddH20.

Primers used for PCR and sequencing

PCR was performed using 5µl of DNA (3ng/µl) 12.5µl 2X LongAmp (NEB) with 1.25µl of each 10µM MGC-ITS3 and MGC-ITS3 primer (MGC-ITS3; TACACGACGTTGTAAAACGACGCATCGATGAAGAACGCAGC) and (MGC-ITS3R; AGGATAACAATTTCACACAGGATTTGAGCTCTTGCCGCTTCA) with 10µl ddH20 for a 25µl total reaction. An initial 95°C 5 minute denaturization was performed followed by 40 cycles of 95°C for 15s and 65°C for 90s. Samples were purified with 75µl SenSATIVAx, washed twice with 100µl 70% EtOH and bench dried for 5 minutes at room temperature. Samples were eluted in 25µl ddH20.

Total Yeast and Mold assay and ITS amplification

A commercially available total yeast and mold qPCR assay (TYM-PathogINDICAtor, Medicinal Genomics, Woburn MA) was used to screen for fungal DNA in a background of host *Cannabis* DNA. The TYM qPCR assay targets the ribosomal DNA Internal Transcribed Spacer region 2 (ITS2) using modified primers described previously[27,28]. Fungal DNA amplified using these primers may also be subjected to next generation sequencing to identify the contributing yeast and mold species. ITS sequencing has been widely used to identify and enumerate fungal species present in a given sample[29].

Tailed PCR cloning and sequencing

DNA libraries were constructed with 250ng DNA using New England Biolabs (Ipswich, MA) NEBNext Quick ligation module (NEB # E6056S). End repair used 3µl of enzyme mix, 6.5µl of reagent mix, 55.5µl of DNA + ddH20. Reaction was incubated at 30°C for 20 minutes. After end repair, ligation was performed directly with 15µl of blunt end TA mix, 2.5µl of Illumina adaptor (10µM) and 1µl of ligation enhancer (assumed to be 20% PEG 6000). After 15 minute ligation at 25°C, 3µl of USER enzyme was added to digest the hairpin adaptors and prepare for PCR. The USER enzyme was tip-mixed and incubated at 37°C for 20 minutes. After USER digestion, 86.5µl of SenSATIVAx was added and mixed. The samples were placed on a magnet for 15 minutes until the beads cleared and the supernatant could be removed. Beads were washed twice with 150µl of 70% EtOH. Beads were left for 10 minute to air dry and then eluted in 25µl of 10mM Tris-HCl.

Library PCR

25µl 2X Q5 polymerase was added to 23µl of DNA with 1µl of i7 index primer (25µM) and 1µl universal primer (25µm). After an initial 95°C for 10s, the library was amplified for 15 cycles of 95°C 10s, 65°C 90s. Samples were purified by mixing 75µl of SenSATIVAx into the PCR reaction. The samples were placed on a magnet for 15 minutes until the beads cleared and the supernatant could be removed. Beads were washed twice with 150µl of 70% EtOH. Beads were left for 10 minute to air dry and then eluted in 25µl of 10mM Tris-HCl. Samples were prepared for sequencing on the MiSeq version 2 chemistry according to the manufacturers' instructions. 2×250bp reads were selected to obtain maximal ITS sequence information.

PaxP verification PCR

Primers described by Shirazi-zand *et al*. were utilized to amplify a segment of the 725bp *PaxP* gene from *Penicillium paxilli*. 25µl LongAmp (NEB) 4µl 10µM primer, 1µl DNA (14ng/µl), 20µl ddH20 to make a 50µl PCR reaction. Cycling conditions were slightly modified to accommodate a different polymerase. 95°C for 30s followed by 28 cycles of 95°C 15s, 55°C for 30s, 65°C 2.5 minutes. Samples were purified with 50µl of SenSATIVAx as described above. 1µl of purified PCR product was sized on Agilent HS 2000 chip. Nextera libraries and sequencing were performed according to instructions from Illumina using 2×75bp sequencing on a version 2 MiSeq.

Penicillium Citrinum verification PCR

Citrinum forward GATTTTCCAAAATGCCGTCT and Citrinum reverse GCTCAAGCATTAATCTAGCTA primers were used with identical PCR conditions as above with the exception using 35 cycles of PCR. Samples were purified with 50µl of SenSATIVAx as described above. 1µl of purified PCR product was sized on Agilent HS 2000 chip. Nextera libraries and sequencing were performed according to instructions from Illumina using 2×75bp sequencing on a version 2 MiSeq. Reads were mapped to Genbank accession number LKUP01000000. Mappings were confirmed using BLAST to NCBI to ensure the strongest hits were to *P. citrinum*.

Analysis

Reads were demultiplexed and trimmed with Casava 1.8.2 and trim_galore v0.4.1 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). FLASH v1.2.11[30] was used to merge the reads using max_overlap 150. The reads were aligned to microbial references using MG-RAST v3.2[31]. Alignments and classifications were confirmed with a second software tool from One Codex (https://onecodex.com/) and critical pathways identified for further evaluation with PCR of toxin producing genes. Reads are deposited in NCBI under SRA accession: SRP065410. Nextera 2×75bp sequencing of the *PaxP* gene was mapped to accession number HM171111.1 with CLCbio Workstation V4 at 98% identity over 80% of the read. One Codex analysis was put into Public mode under the following public URLs:

Australian Bastard:
https://app.onecodex.com/analysis/public/201e7f1642e04a3c
https://app.onecodex.com/analysis/public/58f1e03c10434bfa

KD4:
https://app.onecodex.com/analysis/public/2e86e262817246c4
https://app.onecodex.com/analysis/public/1abd5b60446140a0

KD6:
https://app.onecodex.com/analysis/public/a92d3dff5485499d
https://app.onecodex.com/analysis/public/8d72e2514e564ecd

KD8:
https://app.onecodex.com/analysis/public/8d72e2514e564ecd
https://app.onecodex.com/analysis/public/d6e2e0bcfba3469f

Liberty Haze:
https://app.onecodex.com/analysis/public/7bcd650fa5544f2c
https://app.onecodex.com/analysis/public/7f0feb6cb0a94d56

Girls Scout Cookie:
https://app.onecodex.com/analysis/public/a71b1ce8331c461d
https://app.onecodex.com/analysis/public/8d6f10c7ee684f93

Jakes Grape:
https://app.onecodex.com/analysis/public/bc8af5ed19e5407a
https://app.onecodex.com/analysis/public/99d7a4a2f7af486b

RECON:
https://app.onecodex.com/analysis/public/8a22a16cc2e24731
https://app.onecodex.com/analysis/public/0af6ae26a01f48d5

GreenCrack:
https://app.onecodex.com/analysis/public/6114843d2eb3425e
https://app.onecodex.com/analysis/public/3eee642786c54a88

LA Confidential:
https://app.onecodex.com/analysis/public/01e8aefb0d4f4f62
https://app.onecodex.com/analysis/public/b74c2988fcd84e38

NYC Diesel:
https://app.onecodex.com/analysis/public/441cfad759f64dcc
https://app.onecodex.com/analysis/public/d97b39cae96c4a44

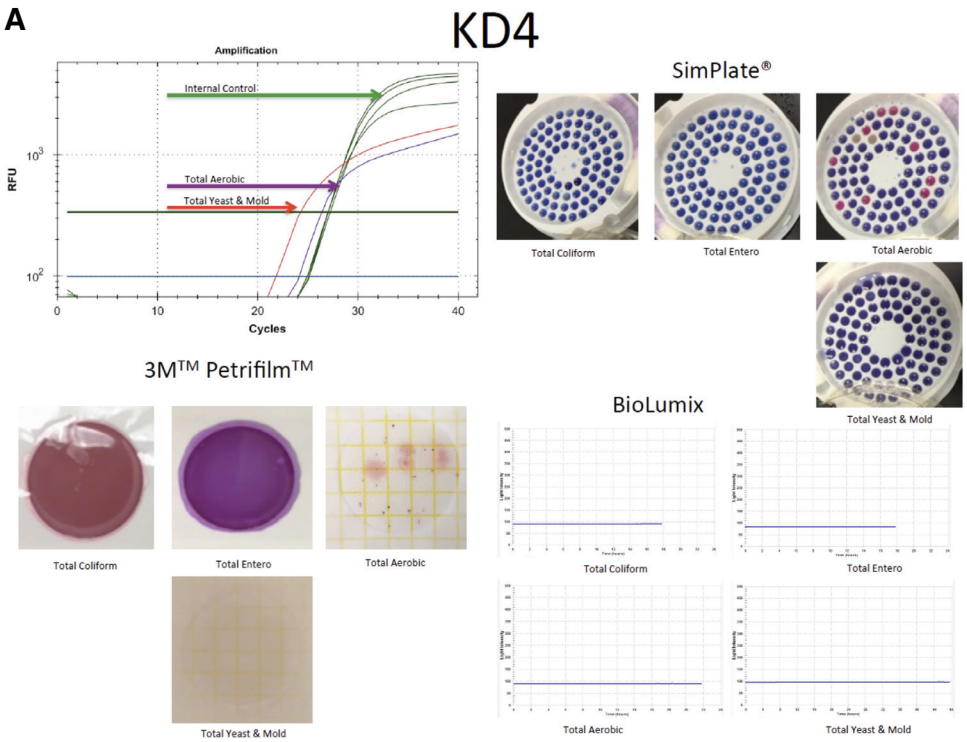
Results

We purified DNA from *Cannabis* samples obtained from two different geographic regions (Amsterdam and Massachusetts) several years apart (2011 and 2015). The majority of samples purified and screened with ITS qPCR were negative for amplification signal implying reagents clean of fungal contamination. Six of the 17 dispensary-derived *Cannabis* samples tested positive for yeast and mold in the TYM qPCR assay. These results were compared with the results derived from three commercially available culture-based detection systems for each of the 17 samples (3M Petrifilm™ 3M Microbiology, St. Paul, MN, USA; SimPlates™ Biocontrol Systems, Bellevue, WA, USA; BioLumix™ Neogen, Lansing MI, USA; Figure 1). Of the 6 qPCR positive samples, two tested negative in all 3 culture-based assays and four tested negative in 1 or 2 of the culture-based assays (Table 1). None of the qPCR negative samples tested positive in any of the culture-based assays. Each of the 6 discordant samples was subjected to ITS sequencing to precisely identify the collection of microbes present. Four additional samples from a different geographic origin (Amsterdam) were also subjected to ITS sequencing, for a total of 10 *Cannabis* samples.

Each discordant sample presented with an array of microbial species, as shown in Figure 2. No sample presented with a single dominant species, and each sample displayed multiple species of interest. Of particular concern were the identified DNA sequences from toxin producing species: *Aspergillus versicolor*[32–36], *Aspergillus terreus*[37], *Penicillium citrinum*[38–40], *Penicillium paxilli*[41,42].

We further analyzed the ITS sequence alignments using the whole genome shotgun based microbiome classification software known as One Codex[43]. Nine of the ten samples sequenced showed the presence of *P. paxilli* (Figure 3). To verify the accuracy of this ITS phylotyping, a gene involved in the paxilline toxin biosynthesis pathway of *P. paxilli* was amplified with PaxPss1 and PaxPss2 primers described by Saikia *et al.*[44]. The resulting 725bp amplicon (expected size) was sequenced to confirm the presence of the *P. paxilli* biosynthesis gene in the *Cannabis* sample KD8 (Figure 4). This was successfully repeated with primers designed to target genes in the citrinin pathway of *P. citrinum*. There were some discrepancies between the results derived from the two software platforms (One Codex and MG-RAST). The MG-RAST analysis, using merged, paired reads correlated better with the PCR results. While One Codex predicted and confirmed KD8 as having the highest *P. paxilli* content, the One Codex platform is optimized for whole genome shotgun data and may not be able to differentiate the 18S sequence differences (391/412 aligned bases) between these two species with a K-mer based approach.

With the confirmed presence of *P. paxilli*, we are curious to find out whether the toxin, paxilline, is present in the samples. Development of monoclonal antibodies to paxilline has recently been described[45], but commercial ELISA assays with sensitivity under 50ppb do not appear to be available at this time. A >50ppb multiplexed ELISA assay is available from Randox Food Diagnostics (Crumlin, UK). Detection with LC-MS/MS has also been described[46,47], however, and experiments are underway to determine whether paxilline can be identified in the background of cannabinoids and terpenes present in *Cannabis* samples.



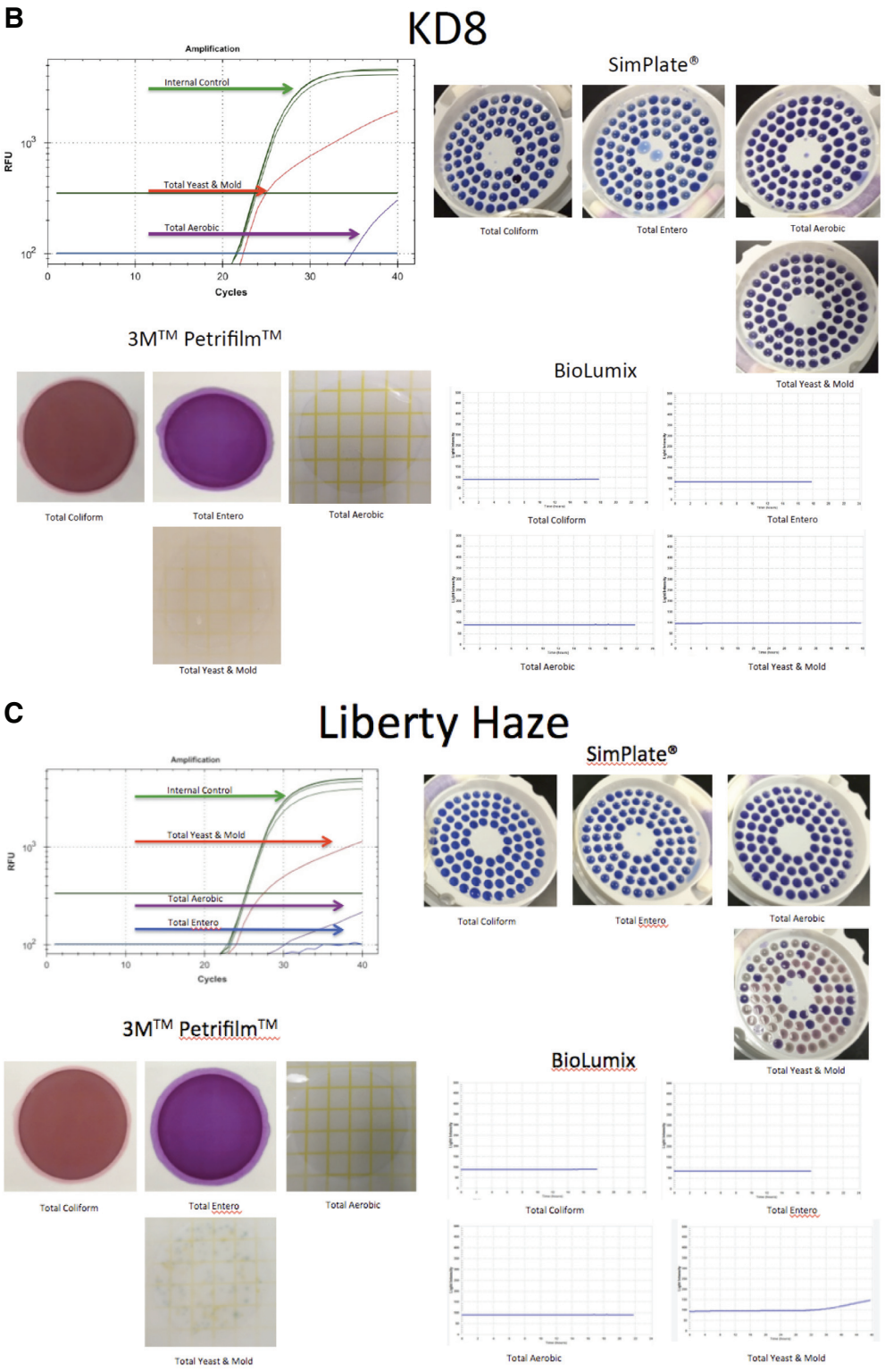


Figure 1. Comparison of 4 different microbial detection technologies. Figure 1A. qPCR signal from TYM (red line) test run concurrently (multiplexed) with a plant internal control marker (green line). This marker targets a conserved region in the *Cannabis* genome and should show up in every assay (upper 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 tests (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.

Table 1. Samples were cultured with 3 different techniques and compared to quantitative PCR (qPCR). Biolumix had the lowest sensitivity failing to pick up 4/17 samples detected with other culture-based platforms. qPCR identified 2 samples that were not picked up 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. The raw CFU numbers can be deduced by dividing the CFU number by the 1,000 fold dilution factor used in this study.

Samples	Total Yeast and Mold (10,000 CFU/g = fail)			
	Simplate® (CFU/g)	3M® (CFU/g)	BioLumix® (CFU/g)	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 -spiked	>738,000 (f)	TNTC (f)	fail	15.71 (f)
Girl Scout Cookies	>738,000 (f)	TNTC (f)	pass	19.66 (f)
Jake's Grape	>738,000 (f)	TNTC (f)	pass	24.56 (f)
Serious Happiness	0	0	pass	>40 (p)
White Rhino	0	3000 (p)	pass	>40 (p)

TNTC = Too Numerous To Count

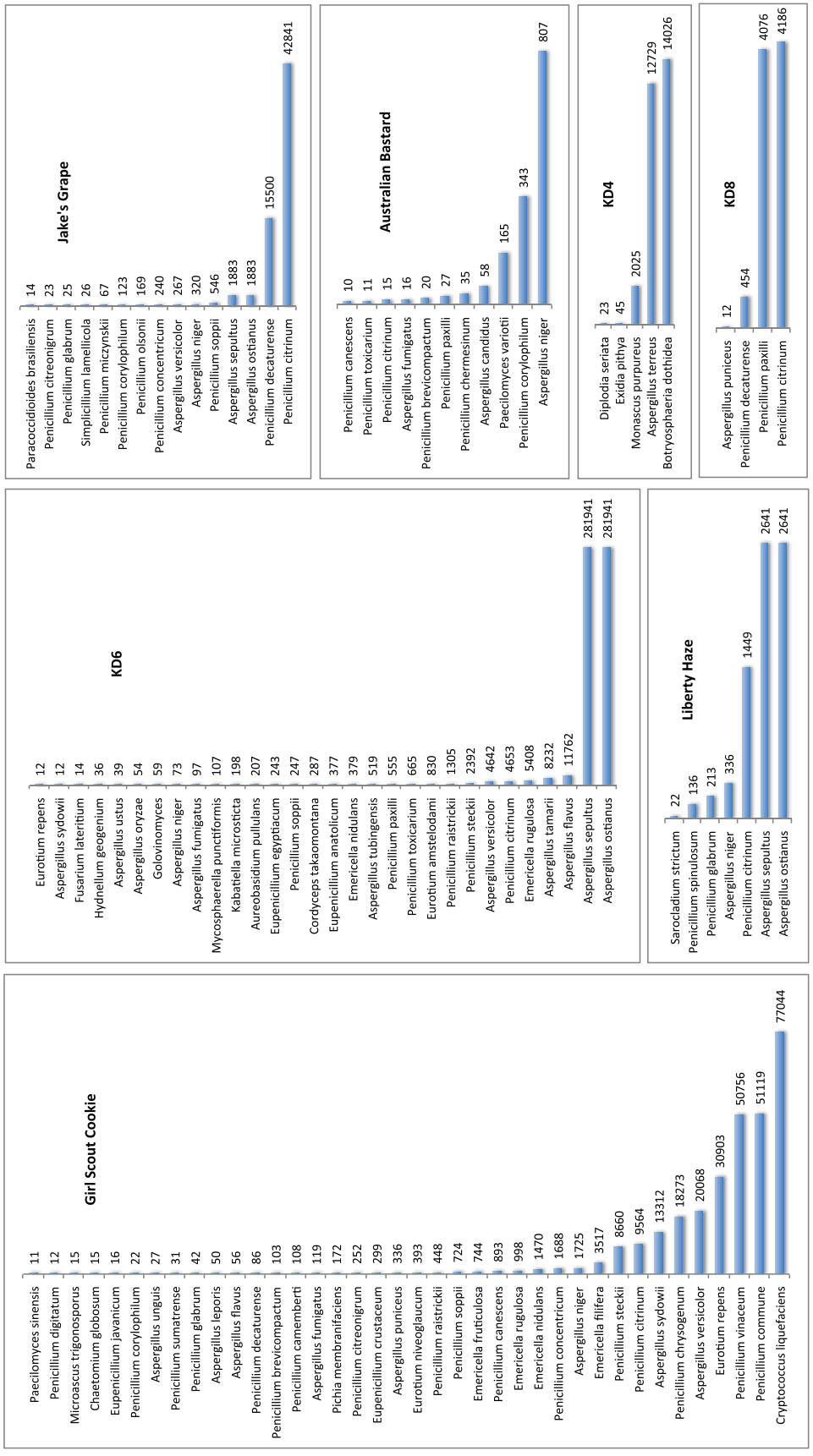


Figure 2. Detection of fungal species by ITS2 sequencing and MG-RAST analysis. Histograms are provided for each of the *Cannabis* samples that tested negative by at least one culture based method and positive using a qPCR-based total yeast and mold test. The number of reads corresponding to each detected fungal species is indicated to the right of each bar. Species detected with less than 10 reads are not included. The overall read counts per sample are more a reflection of sample normalization for sequencing than of the absolute fungal DNA levels.

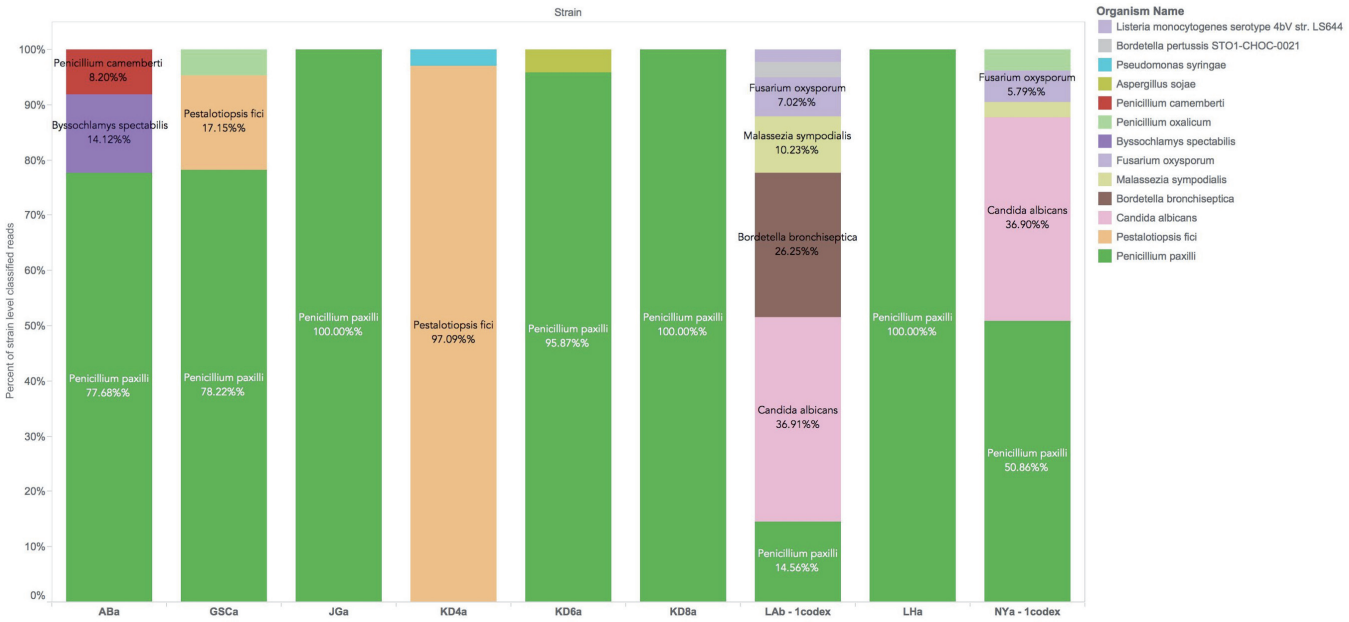


Figure 3. One Codex classification of ITS reads. *P. paxilli* is the most frequently found contaminant in *Cannabis* flowers. *P. citrinum* is not in the One Codex database at this time. One Codex utilizes a fast k-mer based approach for whole genome shotgun classification and can be influenced by read 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.

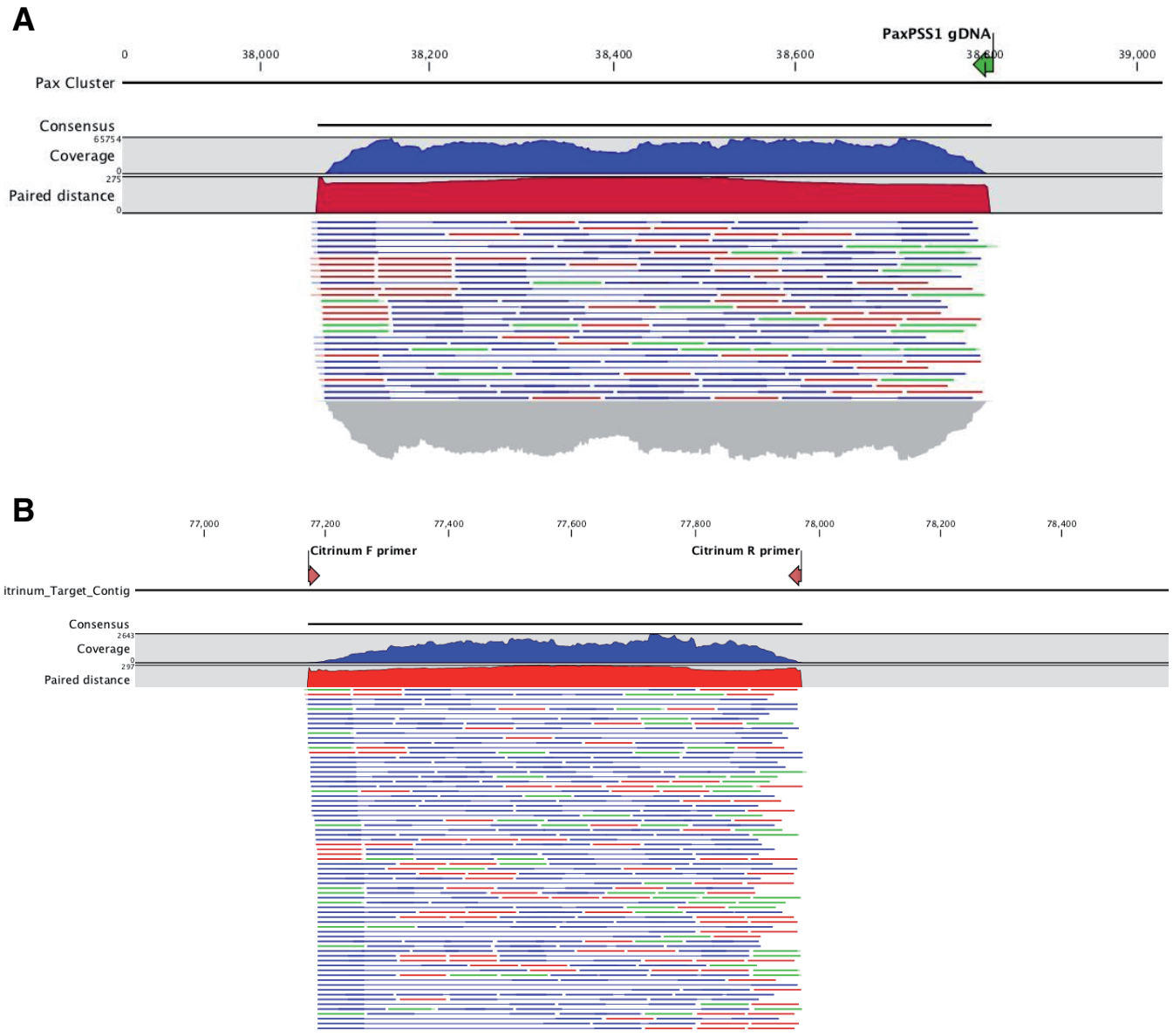


Figure 4. PCR of genes encoding Paxilline and Citrinin demonstrates amplification of the expected size. Citrinum primers we designed from Genbank accession number LKUP01000753. Paxilline primers were used as described in Saikia *et al.* PCR products were made into shotgun libraries with Nextera and sequenced on an Illumina MiSeq with 2×75bp reads to over 10,000X coverage. Reads were mapped with CLCbio 4 to NCBI accession number HM171111.1 (**A**) and LKUP01000000 respectively (**B**). Paired reads are displayed as blue lines, green and red lines are unpaired reads. Read coverage over the amplicons are 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. *P. paxilli* mappings are displayed on top (**A**) and *P. citrinum* mappings are displayed on bottom (**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.*

Discussion

This study demonstrates detection of numerous fungal species by molecular screening of ITS2 in several dispensary-derived *Cannabis* samples. These included the toxigenic *Penicillium* species: *P. paxilli, P. citrinum*, *P. commune, P. chrysogenum, P. corylophilum, Aspergillus* species: *A. terreus, A. niger, A. flavus*, *A. versicolor* and *Eurotium repens.* In addition, a pathogenic species *Cryptococcus liquefaciens* was detected. The fungal microbiomes of the different samples differed significantly in the number and diversity of species present. Two samples contained a large diversity of species, similar to previous studies that used field-grown samples and culture-based outgrowth methods[2,3,48]. Other samples contained only a few species at significant levels. This is perhaps not surprising given the prevalence of indoor culture methods using artificial growth media for medicinal *Cannabis*. However, we do not have any knowledge of the specific growth conditions that were used for the samples analyzed.

Three different culture-based assays failed to detect all of the positive samples and one, BioLumix™, detected only one out of 7 positive samples. A review of the literature suggests that *Penicillium* microbes can be cultured on CYA media, but some may require colder temperatures (21-24C) and 7 day growth times[49]. Of the *Penicillium,* only *P. citrinum* has been previously reported to culture with 3M Petri-Film[50]. It is possible the different water activity of the culture assay compared to the natural flower environment is contributing to the false negative test results.

Quantitative PCR is agnostic to water activity and can be performed in hours instead of days. The specificity and sensitivity provides important information on samples that present risks invisible to culture based systems. The drawback to qPCR is the method's indifference to living or non-living DNA. While techniques exist to perform live-dead qPCR, the live status of the microbes is unrelated to toxin potentially produced while the microbes were alive. ELISA assays exist to screen for some toxins[51]. Current state-recommended ELISA's do not detect citrinin or paxilline, the toxins produced by *P. citrinum* and *P. paxilli*, respectively. The predominance of these *Penicillium* species in a majority of the samples tested is interesting. Several *Penicillium* species are known to be endophytes on various plant species, including *P. citrinum*[18], and this raises the question of whether they may be common *Cannabis* endophytes. Indeed, *P. citrinum* and a species identified as *P. copticola* (a member of the citrinun section[51]) have previously been identified as *Cannabis* endophytes, along with several *Aspergillus* species[2,3].

Paxilline is a tremorgenic and ataxic potassium channel blocker and has been shown to attenuate the anti-seizure properties of cannabidiol in certain mouse models[52–54]. Paxilline is reported to have tremorgenic effects at nanomolar concentrations and is responsible for Ryegrass-staggers disease[55]. Cannabidiol is often used at micromolar concentrations for seizure reduction and contamination with paxilline, if confirmed, would be a cause for concern. Citrinin is a mycotoxin that disrupts Ca2+ efflux in the mitochondrial permeability transition pore (mPTP)[56–63]. Ryan *et al.* demonstrated that cannabidiol affects this pathway suggesting a similar potential cause for concern regarding CBD-citrinin interaction[64]. Considering the hydrophobicity of these mycotoxins and the growing interest in the use of extracted oils from CBD-rich *Cannabis* strains for treatment of drug resistant epilepsy[65–70], more precise molecular screening of fungal toxins in these products might be warranted.

ITS amplification and sequencing offers a hypothesis-free testing approach that can be employed to identify a broad range of fungal species present in a given sample. Appropriate primer design can survey a broad spectrum of fungal genomes while affording rapid iteration of design. Quantitative PCR has also demonstrated single molecule sensitivity and linear dynamic range over 5 orders of magnitude offering a very sensitive approach for detection of microbial risks. Our survey of *Cannabis* flowers in this study was limited, however. Further studies are required to survey a broader range of samples, and to determine whether paxilline, citrinin, aflatoxin or ochratoxin can be detected at concentrations that represent a clinical risk in *Cannabis* samples or extracts derived from plants that test positive for the fungi known to produce those toxins.

Conclusions

Several toxigenic fungi were detected in dispensary-derived *Cannabis* samples using molecular amplification and sequencing techniques. These microbes were not detected using traditional culture-based platforms. These results suggest that culture based techniques borrowed from the food industry should be re-evaluated for *Cannabis* testing to ensure that they are capable of detecting the prevalent species detected by molecular methods with adequate sensitivity. We recommend that additional sequencing studies be performed to characterize the fungal and bacterial microbiomes of a more diverse selection of *Cannabis* samples. Such sampling should include dispensary-derived samples from both indoor and outdoor crops, as well as samples from police seizures from well-provenanced foreign sources, such as Mexico. Finally, further studies should be performed to measure toxin levels in strains that test positive for toxigenic species.

Author contributions

KJM designed the study and performed the One-Codex analysis and PCR verifications. JS designed and ran the culture and qPCR laboratory experiments. LZ assisted in the figure generation and laboratory experiment. YH assisted in sequencing and PCR confirmation of Pax. VT- read alignment, MG-RAST, primer design and analysis. TF- Sample tracking software, figure generations, ITS software comparisons. DS- Manuscript construction and review.

Competing interests

The authors are employees of Medicinal Genomics Corporation (MGC). MGC manufactures qPCR reagents utilized in this study.

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Current Referee Status: ✔ ✔

Version 2

Referee Report 06 June 2016

doi:10.5256/f1000research.9041.r13979

✔ **Donald Cooper**
Chief Science Officer, Mobile Assay Inc., Boulder, CO, USA

The authors have adequately addressed most of my concerns with the exception of the issue of re-evaluating the standard methods used for establishing food safety thresholds as applied to cannabis.

The problem is with the use of the word "re-evaluate". The authors are suggesting that the negative results they obtained using cell culture-based platforms in samples that were positive using their more sensitive molecular amplification indicate a possible limitation of standard methods as applied to cannabis. This may or may not be true. The concern I have is whether their technique is overly sensitive based on current limits used in the food industry. It may be that use of molecular DNA amplification methods indicate the presence of several toxigenic species of fungi and other pathogens in food but at such low levels that standard culture-based methods would not detect them. In this case there would be no need to re-evaluate the use of standard methods that establish safety thresholds. In short, without some type of calibration between the author's technique and traditional methods there is no way of knowing whether their sensitivity is too high or traditional methods used in the food industry are somehow not capable of detecting pathogens in cannabis preparations and therefore need to be "re-evaluated".

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Referee Report 04 February 2016

doi:10.5256/f1000research.8086.r11800

? **Donald Cooper**
Chief Science Officer, Mobile Assay Inc., Boulder, CO, USA

The manuscript is well written and appropriate as an article in F1000 Research. The abstract states that their findings, "demonstrate the limitations in the culture-based regulations", but this conclusion does not follow from their data. In fact, their results show that their DNA based method is overly sensitive at

detecting potential pathogens. Whether culture-based regulations are appropriate or not would have to be validated and tested directly, not simply inferred from the presence of microbial DNA. The authors should remove this cautionary sentence in the abstract and throughout the manuscript until it has been validated. This would make the manuscript more balanced and justified.

A better understanding of the microbiome and mycobiome in cannabis is an important endeavor in part, because recent work on the human microbiome has revealed that microbial constituents of the microbiome and fungi interact cooperatively and non cooperatively to influence human health. Recently studies focused on the human gut mycobiome have been performed using deep sequencing of the ITS1 region for identification of fungi in fecal samples from healthy individuals and the researchers identified 184 fungal species in total. Human oral and lung tissues testing indicate the presence of *Aspergillus, Cryptococcus, Fusarium*, and *Alternaria* in healthy individuals. An emerging theme from this new field of study indicates complex microbial communities distributed across the body that fundamentally contribute to the development, physiology and metabolic homeostasis of the macro-organism. The same is likely true in plants, like cannabis. Because of this beneficial dynamic interplay between microbes and hosts a complete absence of amplified DNA or RNA microbial markers would be unexpected. The question is, “What levels are safe?” and the answer to this question has not been established.

From a consumer safety perspective the rationale for microbial testing in food and pharmaceuticals is to prevent infection of highly toxigenic microbes that grow readily and are consumed in rather large quantities. For example, *Aspergillus flavus* infects grain in as much as 30% of Sub-Saharan African maize and the Aflatoxin regulatory limit for maize is 10 ppb because in this region maize is consumed in kilogram quantities on a daily basis. The risk of health hazard to individuals arising from microbial toxins or mycotoxins is largely proportional to the consumption amounts and frequency. Compared to other regulated foods or tobacco it is expected that the level of cannabis consumption would be minimal and would therefore present minimal risk, nevertheless, there is burgeoning interest in putting in place regulatory requirements for medical and recreational Cannabis in some markets.

In this present study the authors use state-of-the art technology to identify DNA-based markers associated with a variety of microbes and, as expected, some are pathogenic. The authors state that “Mycotoxin monitoring in Cannabis preparations is important since aflatoxin produced by *Aspergillus* species is a carcinogen.”, but there is no actual data showing that the strain of *Aspergillus* they identify is toxigenic. *Aspergillus* is a common fungus that is found in the human mycobiome in oral, lung, gastrointestinal tract. Detection of *Aspergillus* markers in minute quantities alone is not necessarily a health concern unless it is also coincident with live cells that can grow readily and secrete toxin that are above some threshold or there is an imbalance in homeostatic growth limiting factors. The authors call into question culture-based testing, which is the standard in Food safety and USP regulatory guidelines based on comparison to their highly sensitive DNA based detection. Their results using standard regulatory methods, for the most part, would not indicate a food safety problem. The authors should add this emphasis. The authors correctly identify a limitation of their study in that the qPCR based testing has an “indifference to living or non-living DNA” and because of this their PCR based approach may be unnecessarily sensitive. To date there are little to no guidelines for thresholds on many mycotoxins or bacterial toxins that have been established in the cannabis industry, so their findings help inform regulators as to which type of toxins might be relevant for further analysis.

The authors state, ”Health compromised patients exposed to aflatoxin and clearance-inhibiting cannabinoids raise new questions in regards to the current safety tolerances to aflatoxin.” but the authors present no data showing the presence of aflatoxin in any of their samples. So their caution is highly speculative and they should indicate that unless they provide data supporting their caution.

As mentioned above, dynamic homeostatic processes limit the growth of microbes and fungi in living organisms and the authors point out that “several studies have demonstrated plant phytochemicals and terpenoids like eugenol can inhibit the growth of fungi. It is possible the different water activity of the culture assay compared to the natural terpene rich flower environment is contributing to the false negative test results.” but these phytochemicals may also prevent the growth of fungi and bacteria in the plant despite the presence of microbial DNA.

Furthermore they state that “While techniques exist to perform live-dead qPCR, the live status of the microbes is unrelated to toxin potentially produced while the microbes were alive. ELISA assays exist to screen for some toxins. Current state-recommended ELISA’s do not detect citrinin or paxilline, the toxins produced by P. citrinum and P. paxilli, respectively. The predominance of these Penicillium species in a majority of the samples tested is interesting.” Ideally the authors would test for these toxins in their most positive samples.

The authors state that, “Cannabidiol is often used at micromolar concentrations for seizure reduction implying sub-percentage contamination of paxilline could still be a concern” but this is highly speculative and the authors should de-emphasize the “concern” and state instead that if their results were verified by tests indicating high levels of paxilline then it may be cause for concern. The same is true for their concern about Citrinin and aflatoxin and the authors should state this.

The authors state, “While ELISA assays are easy point of use tests that can be used to detect fungal toxins, they can suffer from lack of sensitivity and cross reactivity. ITS amplification and sequencing offers hypothesis-free testing that can complement the lack of specificity in ELISA assays.” ELISA and rapid diagnostic lateral flow tests are standard in the food safety industry for measuring toxins. I see no need to call into question protein based ELISA methods without even testing them in the first place. All diagnostic tests have sensitivity and selectivity limitations which is why they need to be tested and verified using other analytical methods.

The authors state that “Appropriate primer design can survey a broad spectrum of microbial genomes while affording rapid iteration of design. Quantitative PCR has also demonstrated single molecule sensitivity and linear dynamic range over 5 orders of magnitude offering a very robust approach for detection of microbial risks. This may be important for the detection of nanomolar potency mycotoxins”. The ability to detect single copies of genes makes their system highly sensitive, but does not indicate level of mycotoxin. The authors should point out the limitations of their approach and discuss the possibility that it would likely generate a high degree of false positive results compared to culture-based standard methods.

The authors state, “These results demonstrate that culture based techniques superimposed from the food industry should be re-evaluated based on the known microbiome of actual Cannabis flowers in circulation at dispensaries.” This statement appears to be too strong in light of their data. Without validation for the presence of toxins above a safety threshold there is no need to re-evaluate the standard methods in the food industry.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 17 Mar 2016

Kevin Mckernan, Medicinal Genomics Corporation, USA

The manuscript is well written and appropriate as an article in F1000 Research. The abstract states that their findings, "demonstrate the limitations in the culture-based regulations", but this conclusion does not follow from their data. In fact, their results show that their DNA based method is overly sensitive at detecting potential pathogens. Whether culture-based regulations are appropriate or not would have to be validated and tested directly, not simply inferred from the presence of microbial DNA. The authors should remove this cautionary sentence in the abstract and throughout the manuscript until it has been validated. This would make the manuscript more balanced and justified.

Author response: The last sentence of the abstract was revised to read: Here we describe the first next generation sequencing survey of the fungal communities found in dispensary based *Cannabis* flowers by ITS2 sequencing, and demonstrate the sensitive detection of several toxigenic *Penicillium* and *Aspergillus* species, including *P. citrinum and P. paxilli,* that were not detected by one or more culture-based methods currently in use for safety testing.

A better understanding of the microbiome and mycobiome in cannabis is an important endeavor in part, because recent work on the human microbiome has revealed that microbial constituents of the microbiome and fungi interact cooperatively and non cooperatively to influence human health. Recently studies focused on the human gut mycobiome have been performed using deep sequencing of the ITS1 region for identification of fungi in fecal samples from healthy individuals and the researchers identified 184 fungal species in total. Human oral and lung tissues testing indicate the presence of Aspergillus, Cryptococcus, Fusarium, and Alternaria in healthy individuals. An emerging theme from this new field of study indicates complex microbial communities distributed across the body that fundamentally contribute to the development, physiology and metabolic homeostasis of the macro-organism. The same is likely true in plants, like cannabis. Because of this beneficial dynamic interplay between microbes and hosts a complete absence of amplified DNA or RNA microbial markers would be unexpected. The question is, “What levels are safe?” and the answer to this question has not been established.

From a consumer safety perspective the rationale for microbial testing in food and pharmaceuticals is to prevent infection of highly toxigenic microbes that grow readily and are consumed in rather large quantities. For example, Aspergillus flavusinfects grain in as much as 30% of Sub-Saharan African maize and the Aflatoxin regulatory limit for maize is 10 ppb because in this region maize is consumed in kilogram quantities on a daily basis. The risk of health hazard to individuals arising from microbial toxins or mycotoxins is largely proportional to the consumption amounts and frequency. Compared to other regulated foods or tobacco it is expected that the level of cannabis consumption would be minimal and would therefore present minimal risk, nevertheless, there is burgeoning interest in putting in place regulatory requirements for medical and recreational Cannabis in some markets.

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Author response: We thank the reviewer for the background and context provided to his concern. The questions about what levels of toxins are safe or acceptable fall well beyond the scope of the present study. We concede, however, that some statements made in the introduction may have been too speculative. We have revised paragraph 2 of the introduction to focus on the numerous literature reports of pulmonary aspergillosis associated with cannabis use instead of potential mycotoxin toxicity. We have also provided more background on the overlap of cannabinoid and mycotoxin metabolism via cytochrome P450 system as follows. Monitoring for mycotoxic fungi in cannabis preparations has been recommended as part of routine safety testing by the Cannabis Safety Institute. A major driver for this recommendation has been numerous reported cases of serious or fatal pulmonary Aspergillosis associated with marijuana smoking in immunocompromised patients[4-6]. The major cannabinoids have been shown to be potent inhibitors of several cytochrome P450 enzymes at therapeutic concentrations, including 1A1, 1A2, 1B1 2B6, 2C19, 2D6, 3A4 and 3A5[7]. Some of these enzymes have been implicated in the metabolism of the fungal toxins aflatoxin and ochratoxin[8-10]. This raises questions about potential interactions and appropriate safety tolerances for mycotoxins in patients being treated with cannabinoid therapeutics. In addition, some *Fusarium* species that produce toxins have proven to be difficult to selectively culture with tailored media [6–8] . This is a common problem associated with culture-based systems as carbon sources are not exclusive to certain microbes and only 1% of microbial species are believed to be culturable [9] .

The first sentence of the next paragraph was edited slightly to follow more logically: While the risks of mycotoxic fungal contamination have been well studied in the food markets, the presence of the fungal populations present on *Cannabis* flowers has never been surveyed with next generation sequencing techniques [10–15] .

As mentioned above, dynamic homeostatic processes limit the growth of microbes and fungi in living organisms and the authors point out that "several studies have demonstrated plant phytochemicals and terpenoids like eugenol can inhibit the growth of fungi. It is possible the different water activity of the culture assay compared to the natural terpene rich flower environment is contributing to the false negative test results." but these phytochemicals may also prevent the

growth of fungi and bacteria in the plant despite the presence of microbial DNA.

Author response: Upon reconsideration, we concluded that there is no way to predict how growth of the specific fungi detected might be affected by the terpenoids in Cannabis flowers. Those compounds may inhibit the growth of some fungi, while permitting the growth of others. In any case, the effect of terpenes would not relevant to the ability of the fungi to grow on culture media lacking those compounds. That sentence was deleted and the last sentence of paragraph 1 in the discussion was edited as follows: It is possible the different water activity of the culture assay compared to the natural flower environment is contributing to the false negative test results.

Furthermore they state that "While techniques exist to perform live-dead qPCR, the live status of the microbes is unrelated to toxin potentially produced while the microbes were alive. ELISA assays exist to screen for some toxins. Current state-recommended ELISA's do not detect citrinin or paxilline, the toxins produced by P. citrinum and P. paxilli, respectively. The predominance of these Penicillium species in a majority of the samples tested is interesting." Ideally the authors would test for these toxins in their most positive samples.

Author response: We stated transparently that this needed to be done as a future direction. We don't see it as an essential part of the current publication, which had the goal of simply cataloging the fungal content of dispensary-derived samples.

The authors state that, "Cannabidiol is often used at micromolar concentrations for seizure reduction implying sub-percentage contamination of paxilline could still be a concern" but this is highly speculative and the authors should de-emphasize the "concern" and state instead that if their results were verified by tests indicating high levels of paxilline then it may be cause for concern. The same is true for their concern about Citrinin and aflatoxin and the authors should state this.
We agree. Paragraph 3 of the discussion was edited to address this criticism as follows: Paxilline is a tremorgenic and ataxic potassium channel blocker and has been shown to attenuate the anti-seizure properties of cannabidiol in certain mouse models [44–46] . Paxilline is reported to have tremorgenic effects at nanomolar concentrations and is responsible for Ryegrass-staggers disease [47] . Cannabidiol is often used at micromolar concentrations for seizure reduction and contamination with paxilline, if confirmed, would be a cause for concern. Citrinin is a mycotoxin that disrupts Ca2+ efflux in the mitochondrial permeability transition pore (mPTP) [48–55] . Ryan et al. demonstrated that cannabidiol affects this pathway suggesting a similar potential cause for concern regarding CBD-citrinin interaction [56] . Considering the hydrophobicity of these mycotoxins and the growing interest in the use of extracted oils from CBD-rich Cannabis strains for treatment of drug resistant epilepsy[57–62], more precise molecular screening of fungal toxins in these products might be warranted.

The authors state, "While ELISA assays are easy point of use tests that can be used to detect fungal toxins, they can suffer from lack of sensitivity and cross reactivity. ITS amplification and sequencing offers hypothesis-free testing that can complement the lack of specificity in ELISA assays." ELISA and rapid diagnostic lateral flow tests are standard in the food safety industry for measuring toxins. I see no need to call into question protein based ELISA methods without even testing them in the first place. All diagnostic tests have sensitivity and selectivity limitations which is why they need to be tested and verified using other analytical methods.

The authors state that "Appropriate primer design can survey a broad spectrum of microbial

genomes while affording rapid iteration of design. Quantitative PCR has also demonstrated single molecule sensitivity and linear dynamic range over 5 orders of magnitude offering a very robust approach for detection of microbial risks. This may be important for the detection of nanomolar potency mycotoxins". The ability to detect single copies of genes makes their system highly sensitive, but does not indicate level of mycotoxin. The authors should point out the limitations of their approach and discuss the possibility that it would likely generate a high degree of false positive results compared to culture-based standard methods.

Author response: The comments about ELISA assays was deleted and the paragraph was edited to focus on detection of fungal species, not toxins, as follows. ITS amplification and sequencing offers a hypothesis-free testing approach that can be employed to identify a broad range of fungal species present in a given sample. Appropriate primer design can survey a broad spectrum of fungal genomes while affording rapid iteration of design. Quantitative PCR has also demonstrated single molecule sensitivity and linear dynamic range over 5 orders of magnitude offering a very sensitive approach for detection of microbial risks. Our survey of *Cannabis* flowers in this study was limited, however. Further studies are required to survey a broader range of samples, and to determine whether paxilline, citrinin, aflatoxin or ochratoxin can be detected at concentrations that represent a clinical risk in *Cannabis* samples or extracts derived from plants that test positive for the fungi known to produce those toxins.

The authors state, "These results demonstrate that culture based techniques superimposed from the food industry should be re-evaluated based on the known microbiome of actual Cannabis flowers in circulation at dispensaries." This statement appears to be too strong in light of their data. Without validation for the presence of toxins above a safety threshold there is no need to re-evaluate the standard methods in the food industry.

Author response: We respectfully disagree with this comment of the reviewer. The sentence was taken out of context. We were not trying to suggest that the standard methods in use in the food industry should be re-evaluated for all applications, only the use of those methods for medicinal *Cannabis* testing. The Conclusions paragraph was modified to clarify as follows. Several toxigenic fungi were detected in dispensary-derived *Cannabis* samples using molecular amplification and sequencing techniques. These microbes were not detected using traditional culture-based platforms. These results suggest that culture based techniques borrowed from the food industry should be re-evaluated for *Cannabis* testing to ensure that they are capable of detecting the prevalent species detected by molecular methods with adequate sensitivity. We recommend that additional sequencing studies be performed to characterize the fungal and bacterial microbiomes of a more diverse selection of *Cannabis* samples. Such sampling should include dispensary-derived samples from both indoor and outdoor crops, as well as samples from police seizures from well-provenanced foreign sources, such as Mexico. Finally, further studies should be performed to measure toxin levels in strains that test positive for toxigenic species.

Competing Interests: No competing interests were disclosed.No competing interests were disclosed.

Referee Report 17 December 2015

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John McPartland
Division of Molecular Biology,, GW Pharmaceutical, Salisbury, UK

This succinct, well-written study has two major aims: 1. Utilize NextGen and qPCR to identify microorganisms inhabiting dispensary-sourced *Cannabis* flowers. 2. Compare results from these sequencing techniques to results from traditional culture-based methods. Results from the qPCR survey led to a third aim: confirm the presence of two heretofore unreported mycotoxin-producing fungi on *Cannabis: Penicillium citrinum* and *Penicillium paxilli*.

Five critiques:
1. “Microbiome” appears in the manuscript’s title, so you should cite some literature regarding plant microbiomes in general, as well as *Cannabis*-specific research. Vorholt (2012) and Turner *et al.* (2013) provide general overviews. *Cannabis*-specific microbiome studies (Kusari *et al.* 2013, Gautam *et al.* 2013) generated very different results than yours, and should be discussed. The rhizosphere study by Winston *et al.* (2014) ought to be mentioned, and highlight the complimentary nature of rhizosphere and phyllosphere studies.

The foliar microbiome (*aka*, phyllosphere, as opposed to rhizosphere) can be partitioned into two groups: *epiphytes* live upon the leaf epidermis, and *endophytes* occupy intercellular spaces within the leaf. Culture-based detection systems normally surface-sterilize plant samples, so they assume that cultured organisms are endophytes. NextGen and qPCR should identify both epiphytes and endophytes. Classic epiphytes identified in your study include *Kabatiella (Aureobasidium) microsticta* and *Sarocladium (Acremonium) strictum*.

Phyllosphere organisms may be plant pathogens, and cause disease symptoms; diseased plants should never reach a dispensary. However, phyllosphere organisms may act as symbionts (good for the plant) or commensals (indifferent), and their asymptomatic presence is not easily detected. Nevertheless these cryptic organisms may cause disease in humans. The spores from phyllosphere fungi readily pass through waterpipes (Moody *et al.* 1982), and survive in smoke drawn from cannabis cigarettes (Kurup *et al.* 1983), as do aflatoxins (Llewellyn and O'Rear 1977). Worth mentioning.

2. Explain the methodology used to select three culture-based detection systems in this study. Are they the most widely-used systems? Are they the systems recommended by cannabis regulatory agencies? If the method was simply “convenience sampling,” say so.

3. Methods used in the qPCR ITS assay should be described in the Methods section, not the Results section.

4. Figure 2, “DNA sequencing of ITS2 amplicons from culture negative samples that are qPCR positive for total yeast and mold tests,” deserves some comment. Some of the taxa are not yeasts or molds. They include angiosperms *(Zea mays, Pachysandra procumbens*), a protozoan (*Sterkiella histriomuscorum*), and an “uncultured bacterium.” Comment please.

5. This study revealed a surprisingly depauperate *Cannabis* foliar microbiome, compared to a recent study of *Genlisea* species, using similar methods, that identified 92 genera of organisms (Cao *et al.* 2015). See Delmotte *et al.* (2009) for rich microbiomes in other plant species. Gzebenyuk (1984) isolated 79 species of fungi from hemp stems in Russia. Comment please.

Where are the bacteria? Much of the concern over microbiology and food safety focuses on human enteric pathogens (*e.g., Escherichia coli, Salmonella* spp.) and opportunistic bacteria (*e.g., Pseudomonas aeruginosa, Berkholderia cepacia*). Previous culture-based studies have isolated these organisms from *Cannabis* (*e.g.,* Taylor *et al.* 1982, Ungerleider *et al.* 1982). The only bacterium you identify (in Figure 2,

erroneously identified as a yeast or mold) is *Pantoea agglomerans,* formerly known as *Enterobacter agglomerans,* a gram-negative bacterium and an opportunistic human pathogen.

Minor critiques:

1. The detection and confirmation of *Penicillium citrinum* and *Penicillium paxilli* deserves mention in the abstract of the paper! An exhaustive review of the *Cannabis* literature (McPartland *et al.* 2000) found no references to these organisms.
2. Species names should never be capitalized. For example, *P. Citrinum* should read *P. citrinum*.
3. The full name should be spelled out the first time it appears in the Methods section, *Penicillium citrinum*.
4. The Methods section should explain that the *PaxP* gene comes from *Penicillium paxilli.* The Methods section should identify NEP as New England BioLabs.
5. Recommend some future directions: a comparison of indoor crops and outdoor crops (outdoor crops may show a seasonal community succession), and survey the microbiome of police seizures from well-provenanced foreign sources, such as Mexico.

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Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 17 Mar 2016

Kevin Mckernan, Medicinal Genomics Corporation, USA

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Author response: Several of the references cited relate to analysis of the bacterial microbiome on diverse plant species, which falls well beyond the scope of this study (focusing on the fungal microbiome of *Cannabis*). However, we added a brief mention of the very limited literature relating to Cannabis microbiomes to the introduction as follows:

"Our knowledge of the natural microbiome of field-grown *Cannabis* in terms of rhizosphere bacteria, and endophytic fungi is limited to just a few focused studies[1-3]. Very little is known about the potential for bacterial and fungal contamination on medicinal *Cannabis*. Nevertheless, many

states in the U.S. are now crafting regulations for detection of microbial contamination on *Cannabis* in the absence of any comprehensive survey of actual samples. A few of these regulations are inducing growers to “heat kill” or pasteurize *Cannabis* flowers to lower microbial content. While this seems a harmless suggestion, we must remain aware of how these drying techniques may create false negatives in culture-based safety tests used to monitor colony-forming units (CFU). Even though pasteurization may be effective at sterilizing some of the microbial content, it does not eliminate various pathogenic toxins or spores. *Aspergillus* spores and mycotoxins are known to resist pasteurization [1, 2] . Similar thermal resistance has been reported for *E. coli* produced Shiga toxin [3] . While pasteurization may reduce CFU’s used in petri-dish or plating based safety tests, it does not reduce the microbial toxins, spores or DNA encoding these toxins."

Author response: We also added a sentence to the end of the second paragraph of the discussion in reference to the two existing publications on Cannabis endophytic fungi.

"Several *Penicillium* species are known to be endophytes on various plant species, including *P. citrinum* [10] , and this raises the question of whether they are also *Cannabis* endophytes. Indeed, *P. citrinum* and a species identified as *P. copticola* (a member of the citrinun clade[12]) have previously been identified as *Cannabis* endophytes, along with several *Aspergillus* species[2, 3]."

2. Explain the methodology used to select three culture-based detection systems in this study. Are they the most widely-used systems? Are they the systems recommended by cannabis regulatory agencies? If the method was simply “convenience sampling,” say so.

Author response: A sentence was added to the culture based methods section. The culture-based methods selected for testing here represent those currently in use by established medicinal Cannabis safety testing laboratories.

3. Methods used in the qPCR ITS assay should be described in the Methods section, not the Results section.

Author response: Paragraph 1 of the results section was moved to the methods section.

4. Figure 2, “DNA sequencing of ITS2 amplicons from culture negative samples that are qPCR positive for total yeast and mold tests,” deserves some comment. Some of the taxa are not yeasts or molds. They include angiosperms *(Zea mays, Pachysandra procumbens*), a protozoan (*Sterkiella histriomuscorum*), and an “uncultured bacterium.” Comment please.

Author response: The MG-RAST database contains multiple taxa. The hits to non-fungal species contained multiple mismatches and were deemed spurious. We filtered the data to remove all non-fungal hits and regenerated the figures, reporting all fungal species detected with 10 reads or more. This resulted in a higher number of species reported for some samples and fewer for others. Figure 2 was revised and the figure legend expanded.

5. This study revealed a surprisingly depauperate *Cannabis* foliar microbiome, compared to a recent study of *Genlisea*species, using similar methods, that identified 92 genera of organisms (Cao *et al.* 2015). See Delmotte *et al.* (2009) for rich microbiomes in other plant species. Gzebenyuk (1984) isolated 79 species of fungi from hemp stems in Russia. Comment please.

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Author response: This study focuses only on the fungal microbiome. The studies cited are looking at field-grown samples. We revised the MG-RAST figures to include all fungal species detected down to a level of 10 reads. This reveals a significantly larger number of species in two samples. Other species display only a handful of species. Medicinal Cannabis is often grown indoors in artificial media. The first paragraph of the discussion section was expanded and split into two paragraphs as follows:

"This study demonstrates detection of numerous fungal species by molecular screening of ITS2 in several dispensary-derived *Cannabis* samples. These included the toxigenic *Penicillium* species: *P. paxilli, P. citrinum*, *P. commune, P. chrysogenum, P. corylophilum, Aspergillus* species: *A. terreus, A. niger, A. flavus*, *A. versicolor* and *Eurotium repens.* In addition, a pathogenic species *Cryptococcus liquefaciens* was detected. The fungal microbiomes of the different samples differed significantly in the number and diversity of species present. Two samples contained a large diversity of species, similar to previous studies that used field-grown samples and culture-based outgrowth methods[2, 3, 11]. Other samples contained only a few species at significant levels. This is perhaps not surprising given the prevalence of indoor culture methods using artificial growth media for medicinal *Cannabis*. However, we do not have any knowledge of the specific growth conditions that were used for the samples analyzed.
Three different culture-based assays failed to detect all of the positive samples and one, BioLumix [TM], detected only one out of 7 positive samples. A review of the literature suggests that *Penicillium* microbes can be cultured on CYA media, but some may require colder temperatures (21-24C) and 7 day growth times [40] . Of the *Penicillium,* only *P. citrinum* has been previously reported to culture with 3M Petri-Film [41] . It is possible the different water activity of the culture assay compared to the natural flower environment is contributing to the false negative test results."

Minor critiques:
1. The detection and confirmation of *Penicillium citrinum* and *Penicillium paxilli* deserves mention in the abstract of the paper! An exhaustive review of the *Cannabis* literature (McPartland *et al.* 2000) found no references to these organisms.
Here we describe the first next generation sequencing survey of the fungal communities found in dispensary based Cannabis flowers by ITS2 sequencing, and demonstrate the sensitive detection of several toxigenic *Penicillium* and *Aspergillus* species, including *P. citrinum* and *P. paxilli*, that were not detected by one or more culture-based methods currently in use for safety testing.
2. Species names should never be capitalized. For example, *P. Citrinum* should read *P. citrinum*.

fixed
3. The full name should be spelled out the first time it appears in the Methods section, *Penicillium citrinum*.
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4. The Methods section should explain that the *PaxP* gene comes from *Penicillium paxilli.* The Methods section should identify NEP as New England BioLabs.

fixed

5. Recommend some future directions: a comparison of indoor crops and outdoor crops (outdoor crops may show a seasonal community succession), and survey the microbiome of police seizures from well-provenanced foreign sources, such as Mexico.
Added to conclusion paragraph.

Competing Interests: No competing interests were disclosed.