RESEARCH ARTICLE

***Cannabis* microbiome sequencing reveals several mycotoxic fungi native to dispensary grade *Cannabis* flowers [version 1; peer review: 1 approved, 1 approved with reservations]**

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

Medicinal Genomics Corporation, Woburn, MA, USA

V1 First published: 10 Dec 2015, 4:1422
<https://doi.org/10.12688/f1000research.7507.1>
 Latest published: 10 May 2016, 4:1422
<https://doi.org/10.12688/f1000research.7507.2>

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 microbial communities found in dispensary based *Cannabis* flowers and demonstrate the limitations in the culture-based regulations that are being superimposed from the food industry.

Keywords

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

Open Peer Review

Approval Status

	1	2
version 2 (revision) 10 May 2016		view
version 1 10 Dec 2015	view	view

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

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

Any reports and responses or comments on the article can be found at the end of the article.

This article is included in the **Agriculture, Food and Nutrition** gateway.

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

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

Grant information: The author(s) declared that no grants were involved in supporting this work.

Copyright: © 2015 McKernan K *et al.* This is an open access article distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

How to cite this article: McKernan K, Spangler J, Zhang L *et al.* ***Cannabis* microbiome sequencing reveals several mycotoxic fungi native to dispensary grade *Cannabis* flowers [version 1; peer review: 1 approved, 1 approved with reservations]** F1000Research 2015, 4:1422 <https://doi.org/10.12688/f1000research.7507.1>

First published: 10 Dec 2015, 4:1422 <https://doi.org/10.12688/f1000research.7507.1>

Introduction

Many states in the U.S. are crafting regulations for microbial detection on *Cannabis* in absence of any comprehensive survey of *Cannabis* microbiomes. A few of these regulations are inducing growers to “heat kill” or pasteurize *Cannabis* flowers to lower microbial content. While this is a harmless suggestion, we must remain aware of how these drying techniques often 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 toxin³. 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.

Mycotoxin monitoring in *Cannabis* preparations is important since aflatoxin produced by *Aspergillus* species is a carcinogen. The clearance of aflatoxin requires the human liver enzyme CYP3A4 and this liver enzyme is potentially inhibited by cannabinoids^{4,5}. Modern day *Cannabis* flowers can produce up to 25% (w/v) cannabinoids presenting potent inhibition of CYP3A4 and CYP2C19. Health compromised patients exposed to aflatoxin and clearance-inhibiting cannabinoids raise new questions in regards to the current safety tolerances to aflatoxin. Similarly, *Fusarium* species are known to produce fungal toxins and has proven to be difficult to selectively culture with tailored media⁶⁻⁸. This is a common fault of culture-based systems as carbon sources are not exclusive to certain microbes and only 1% of microbial species are believed to be culturable⁹.

While these risks have been well studied in the food markets, the presence of the microbial populations present on *Cannabis* flowers has never been surveyed with next generation sequencing techniques¹⁰⁻¹⁵. With the publication of the *Cannabis* genome^{16,17} and many other pathogenic microbial genomes, quantitative PCR assays have been developed that can accurately quantify fungal DNA present in *Cannabis* samples¹⁸. 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

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 ddH2O.

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; TACACGACGTTGTAAAACGACGCATC-GATGAAGAACGCAGC) and (MGC-ITS3R; AGGATAACAATTTCACACAGGATTGAGCTCTTGCCGCTTCA) with 10µl ddH2O for a 25µl total reaction. An initial 95°C 5 minute denaturation 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 ddH2O.

Tailed PCR cloning and sequencing

DNA libraries were constructed with 250ng DNA using NEB’s NEBNext Quick ligation module (NEB # E6056S). End repair used 3µl of enzyme mix, 6.5µl of reagent mix, 55.5µl of DNA + ddH2O. 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. 2x250bp 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. 25µl LongAmp (NEB) 4µl 10µM primer, 1µl DNA (14ng/µl), 20µl ddH2O 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 2x75bp sequencing on a version 2 MiSeq.

P. 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 2x75bp 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 v1.2.11¹⁹ was used to merge the reads using max_overlap 150. The reads were aligned to microbial references using MG-RAST v3.2²⁰. Alignments and classifications were confirmed with a second software tool from One Codex (<https://one-codex.com/>) and critical pathways identified for further evaluation with PCR of toxin producing genes. Reads are deposited in NCBI under SRA accession: SRP065410. Nextera 2x75bp 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/3eece642786c54a88>

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

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^{21,22}. 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 sample²³.

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.

A

KD4

SimPlate®

3M™ Petrifilm™

BioLumix

B

KD8

SimPlate®

3M™ Petrifilm™

BioLumix

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.

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*^{24–28}, *Aspergillus terreus*²⁹, *Penicillium citrinum*^{30–32}, *Penicillium paxilli*^{33,34}.

We further analyzed the ITS sequence alignments using the whole genome shotgun based microbiome classification software known as One Codex³⁵. 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.*³⁶ 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 described³⁷,

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)			Cq
	Simplate® (CFU/g)	3M® (CFU/g)	BioLumix® (CFU/g)	
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

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^{38,39}, however, and experiments are underway to determine whether paxilline can be identified in the background of cannabinoids and terpenes present in *Cannabis* samples.

Discussion

Several potentially harmful fungal species were detected in dispensary-derived *Cannabis* samples by qPCR and subsequent sequencing in this study. 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 times⁴⁰. Of the *Penicillium*, only *P. citrinum* has been previously reported to culture with 3M Petri-Film⁴¹. In addition, several studies have demonstrated plant phytochemicals and terpenoids like eugenol can inhibit the growth of fungi⁴². 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.

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

Figure 2. DNA sequencing of ITS2 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.

culture based systems. The draw back 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 toxins⁴³. 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*¹⁰, and this raises the question of whether they are also *Cannabis* endophytes.

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

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.

responsible for Ryegrass-staggers disease⁴⁷. Cannabidiol is often used at micromolar concentrations for seizure reduction implying sub-percentage contamination of paxilline could still be a 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 potential concern for CBD-mycotoxin interaction⁵⁶. Considering the hydrophobicity of paxilline and the recent interest in the use of cannabidiol derived from *Cannabis* flower oils for drug resistant epilepsy, more precise molecular screening of fungal toxins may be warranted^{57–62}.

Our survey of *Cannabis* flowers in this study was limited. Further screening will be required to define a set of tests that can adequately capture all risks. 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. 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. Further studies are required to validate better detection methods for these toxins and verify whether paxilline or citrinin are present on *Cannabis* at concentrations that present a clinical risk.

Conclusions

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. Several mycotoxin producing molds were detected that can potentially interfere with the medical use of cannabidiol. These microbes failed to grow on traditional culture-based platforms but were rapidly detected with molecular based techniques. Further studies are required to quantitate the presence and concentration of mycotoxin production.

Figure 4. PCR of genes encoding Paxilline and Citrinin demonstrates amplification of the expected size. Citrinin 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 2x75bp 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.*

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.

Grant information

The author(s) declared that no grants were involved in supporting this work.

Acknowledgments

John McPartland, Cindy Orser, Brad Douglass, Joost Heeroma, Nick Greenfield, Rebecca McKernan and Kellie Dodd for thoughtful advice.

References

- Fujikawa H, Itoh T: **Tailing of thermal inactivation curve of *Aspergillus niger* spores.** *Appl Environ Microbiol.* 1996; **62**(10): 3745–3749.
[PubMed Abstract](#) | [Free Full Text](#)
- Kabak B, Dobson AD: **Biological strategies to counteract the effects of mycotoxins.** *J Food Prot.* 2009; **72**(9): 2006–2016.
[PubMed Abstract](#)
- Rasooly R, Do PM: **Shiga toxin Stx2 is heat-stable and not inactivated by pasteurization.** *Int J Food Microbiol.* 2010; **136**(3): 290–294.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Langouet S, Coles B, Morel F, *et al.*: **Metabolism of aflatoxin B₁ by human hepatocytes in primary culture.** *Adv Exp Med Biol.* 1996; **387**: 439–442.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Yamaori S, Ebisawa J, Okushima Y, *et al.*: **Potent inhibition of human cytochrome P450 3A isoforms by cannabidiol: role of phenolic hydroxyl groups in the resorcinol moiety.** *Life Sci.* 2011; **88**(15–16): 730–736.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Bragulat MR, Martínez E, Castellá G, *et al.*: **Selective efficacy of culture media recommended for isolation and enumeration of *Fusarium* spp.** *J Food Prot.* 2004; **67**(1): 207–211.
[PubMed Abstract](#)
- Castellá G, Bragulat MR, Rubiales MV, *et al.*: **Malachite green agar, a new selective medium for *Fusarium* spp.** *Mycopathologia.* 1997; **137**(3): 173–178.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Desjardins AE, Proctor RH: **Molecular biology of *Fusarium* mycotoxins.** *Int J Food Microbiol.* 2007; **119**(1–2): 47–50.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Stewart EJ: **Growing unculturable bacteria.** *J Bacteriol.* 2012; **194**(16): 4151–4160.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Kusari PKS, Kusari S, Spitteller M, *et al.*: **Endophytic fungi harbored in *Cannabis sativa* L.: diversity and potential as biocontrol agents against host plant-specific phytopathogens.** *Fungal Diversity.* 2013; **60**(1): 137–151.
[Publisher Full Text](#)
- McPartland: **Fungal pathogens of *Cannabis sativa* in Illinois.** *Phytopathology.* 1983; **72**: 797.
- McPartland: **Common names for diseases of *Cannabis sativa* L.** *Plant Dis.* 1991; **75**: 226–227.
- McPartland JM: **Microbiological contaminants of marijuana.** *J Int Hemp Assoc.* 1994; **1**: 41–44.
[Reference Source](#)
- McPartland JM: ***Cannabis* pathogens X: *Phoma*, *Ascochyta* and *Didymella* species.** *Mycologia.* 1994; **86**(6): 870–878.
[Publisher Full Text](#)
- McPartland JM: **A review of *Cannabis* diseases.** *J Int Hemp Assoc.* 1996; **3**(1): 19–23.
[Reference Source](#)
- van Bakel H, Stout JM, Cote AG, *et al.*: **The draft genome and transcriptome of *Cannabis sativa*.** *Genome Biol.* 2011; **12**(10): R102.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- McKernan: **The *Cannabis sativa* Genome.** 2011.
[Reference Source](#)
- McKernan: 2015.
[Reference Source](#)
- Magoč T, Salzberg SL: **FLASH: fast length adjustment of short reads to improve genome assemblies.** *Bioinformatics.* 2011; **27**(21): 2957–2963.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Glass EM, Wilkening J, Wilke A, *et al.*: **Using the metagenomics RAST server (MG-RAST) for analyzing shotgun metagenomes.** *Cold Spring Harb Protoc.* 2010; **2010**(1): pdb prot5368.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Borneman J, Hartin RJ: **PCR primers that amplify fungal rRNA genes from environmental samples.** *Appl Environ Microbiol.* 2000; **66**(10): 4356–4360.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- White TJ, Bruns T, Lee S, *et al.*: **Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics.** *PCR Protocols: A Guide to Methods and Applications.* 1990; 315–322.
[Reference Source](#)
- Mason M, Cheung I, Walker L: **Creating a geospatial database of risks and resources to explore urban adolescent substance use.** *J Prev Interv Community.* 2009; **37**(1): 21–34.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Abd Alla EA, Metwally MM, Mehriz AM, *et al.*: **Sterigmatocystin: incidence, fate and production by *Aspergillus versicolor* in Ras cheese.** *Nahrung.* 1996; **40**(6): 310–313.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Aly SA, Elewa NA: **The effect of Egyptian honeybee propolis on the growth of *Aspergillus versicolor* and sterigmatocystin biosynthesis in Ras cheese.** *J Dairy Res.* 2007; **74**(1): 74–78.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Engelhart S, Looek A, Skutlarek D, *et al.*: **Occurrence of toxigenic *Aspergillus versicolor* isolates and sterigmatocystin in carpet dust from damp indoor environments.** *Appl Environ Microbiol.* 2002; **68**(8): 3886–3890.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Kocić-Tanacković S, Dimić G, Lević J, *et al.*: **Effects of onion (*Allium cepa* L.) and garlic (*Allium sativum* L.) essential oils on the *Aspergillus versicolor* growth and sterigmatocystin production.** *J Food Sci.* 2012; **77**(5): M278–284.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Song F, Ren B, Chen C, *et al.*: **Three new sterigmatocystin analogues from marine-derived fungus *Aspergillus versicolor* MF359.** *Appl Microbiol Biotechnol.* 2014; **98**(8): 3753–3758.
[PubMed Abstract](#) | [Publisher Full Text](#)
- El-Sayed Abdalla A, Zeinab Kheiralla M, Sahab A, *et al.*: ***Aspergillus terreus* and its toxic metabolites as a food contaminant in some Egyptian Bakery products and grains.** *Mycotoxin Res.* 1998; **14**(2): 83–91.
[PubMed Abstract](#) | [Publisher Full Text](#)

30. Ames DD, Wyatt RD, Marks HL, *et al.*: **Effect of citrinin, a mycotoxin produced by *Penicillium citrinum*, on laying hens and young broiler chicks.** *Poult Sci.* 1976; **55**(4): 1294–1301.
[PubMed Abstract](#) | [Publisher Full Text](#)
31. Mazumder PM, Mazumder R, Mazumder A, *et al.*: **Antimicrobial activity of the mycotoxin citrinin obtained from the fungus *Penicillium citrinum*.** *Anc Sci Life.* 2002; **21**(3): 191–197.
[PubMed Abstract](#) | [Free Full Text](#)
32. Park SY, Kim R, Ryu CM, *et al.*: **Citrinin, a mycotoxin from *Penicillium citrinum*, plays a role in inducing motility of *Paenibacillus polymyxa*.** *FEMS Microbiol Ecol.* 2008; **65**(2): 229–237.
[PubMed Abstract](#) | [Publisher Full Text](#)
33. Itoh Y, Johnson R, Scott B: **Integrative transformation of the mycotoxin-producing fungus, *Penicillium paxilli*.** *Curr Genet.* 1994; **25**(6): 508–513.
[PubMed Abstract](#) | [Publisher Full Text](#)
34. Shibayama M, Ooi K, Johnson R, *et al.*: **Suppression of tandem-multimer formation during genetic transformation of the mycotoxin-producing fungus *Penicillium paxilli* by disrupting an orthologue of *Aspergillus nidulans* *uvsC*.** *Curr Genet.* 2002; **42**(1): 59–65.
[PubMed Abstract](#) | [Publisher Full Text](#)
35. Minot SS, Krumm N, Greenfield NB: **One Codex: A Sensitive and Accurate Data Platform for Genomic Microbial Identification.** *bioRxiv.* 2015.
[Publisher Full Text](#)
36. Saikia S, Parker EJ, Koulman A, *et al.*: **Defining paxilline biosynthesis in *Penicillium paxilli*: functional characterization of two cytochrome P450 monooxygenases.** *J Biol Chem.* 2007; **282**(23): 16829–16837.
[PubMed Abstract](#) | [Publisher Full Text](#)
37. Maragos CM: **Development and Evaluation of Monoclonal Antibodies for Paxilline.** *Toxins (Basel).* 2015; **7**(10): 3903–3915.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
38. Vishwanath V, Sulyok M, Labuda R, *et al.*: **Simultaneous determination of 186 fungal and bacterial metabolites in indoor matrices by liquid chromatography/tandem mass spectrometry.** *Anal Bioanal Chem.* 2009; **395**(5): 1355–1372.
[PubMed Abstract](#) | [Publisher Full Text](#)
39. Uhlig S, Egge-Jacobsen W, Vralstad T, *et al.*: **Indole-diterpenoid profiles of *Claviceps paspali* and *Claviceps purpurea* from high-resolution Fourier transform Orbitrap mass spectrometry.** *Rapid Commun Mass Spectrom.* 2014; **28**(14): 1621–1634.
[PubMed Abstract](#) | [Publisher Full Text](#)
40. Houben J, Frisvad JC, Samson RA: **Taxonomy of *Penicillium* section *Citrina*.** *Stud Mycol.* 2011; **70**(1): 53–138.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
41. 3M.
[Reference Source](#)
42. Zare M, Shams-Ghahfarokhi M, Ranjbar-Bahadori S, *et al.*: **Comparative study of the major Iranian cereal cultivars and some selected spices in relation to support *Aspergillus parasiticus* growth and aflatoxin production.** *Iran Biomed J.* 2008; **12**(4): 229–236.
[PubMed Abstract](#)
43. Labs R: 2015.
[Reference Source](#)
44. Shirazi-zand Z, Ahmad-Molaei L, Motamedi F, *et al.*: **The role of potassium BK channels in anticonvulsant effect of cannabidiol in pentylenetetrazole and maximal electroshock models of seizure in mice.** *Epilepsy Behav.* 2013; **28**(1): 1–7.
[PubMed Abstract](#) | [Publisher Full Text](#)
45. Sabater-Vilar M, Nijmeijer S, Fink-Gremmels J: **Genotoxicity assessment of five tremorgenic mycotoxins (fumitremorgen B, paxilline, penitrem A, verruculogen, and verrucosidin) produced by molds isolated from fermented meats.** *J Food Prot.* 2003; **66**(11): 2123–2129.
[PubMed Abstract](#)
46. Sánchez-Pastor E, Andrade F, Sánchez-Pastor JM, *et al.*: **Cannabinoid receptor type 1 activation by arachidonylcyclopropylamide in rat aortic rings causes vasorelaxation involving calcium-activated potassium channel subunit alpha-1 and calcium channel, voltage-dependent, L type, alpha 1C subunit.** *Eur J Pharmacol.* 2014; **729**: 100–106.
[PubMed Abstract](#) | [Publisher Full Text](#)
47. Imlach WL, Finch SC, Dunlop J, *et al.*: **The molecular mechanism of “ryegrass staggers,” a neurological disorder of K⁺ channels.** *J Pharmacol Exp Ther.* 2008; **327**(3): 657–664.
[PubMed Abstract](#) | [Publisher Full Text](#)
48. Chagas GM, Campello AP, Kluppel ML: **Mechanism of citrinin-induced dysfunction of mitochondria. I. Effects on respiration, enzyme activities and membrane potential of renal cortical mitochondria.** *J Appl Toxicol.* 1992; **12**(2): 123–129.
[PubMed Abstract](#) | [Publisher Full Text](#)
49. Chagas GM, Oliveira BM, Campello AP, *et al.*: **Mechanism of citrinin-induced dysfunction of mitochondria. II. Effect on respiration, enzyme activities, and membrane potential of liver mitochondria.** *Cell Biochem Funct.* 1992; **10**(3): 209–216.
[PubMed Abstract](#) | [Publisher Full Text](#)
50. Chagas GM, Oliveira MA, Campello AP, *et al.*: **Mechanism of citrinin-induced dysfunction of mitochondria. IV–Effect on Ca²⁺ transport.** *Cell Biochem Funct.* 1995; **13**(1): 53–59.
[PubMed Abstract](#) | [Publisher Full Text](#)
51. Chagas GM, Oliveira MB, Campello AP, *et al.*: **Mechanism of citrinin-induced dysfunction of mitochondria. III. Effects on renal cortical and liver mitochondrial swelling.** *J Appl Toxicol.* 1995; **15**(2): 91–95.
[PubMed Abstract](#) | [Publisher Full Text](#)
52. Da Lozzo EJ, Oliveira MB, Camieri EG: **Citrinin-induced mitochondrial permeability transition.** *J Biochem Mol Toxicol.* 1998; **12**(5): 291–297.
[PubMed Abstract](#) | [Publisher Full Text](#)
53. Jeswal P: **Citrinin-induced chromosomal abnormalities in the bone marrow cells of *Mus musculus*.** *Cytobios.* 1996; **86**(344): 29–33.
[PubMed Abstract](#)
54. Ribeiro SM, Campello AP, Chagas GM, *et al.*: **Mechanism of citrinin-induced dysfunction of mitochondria. VI. Effect on iron-induced lipid peroxidation of rat liver mitochondria and microsomes.** *Cell Biochem Funct.* 1998; **16**(1): 15–20.
[PubMed Abstract](#) | [Publisher Full Text](#)
55. Ribeiro SM, Chagas GM, Campello AP, *et al.*: **Mechanism of citrinin-induced dysfunction of mitochondria. V. Effect on the homeostasis of the reactive oxygen species.** *Cell Biochem Funct.* 1997; **15**(3): 203–209.
[PubMed Abstract](#) | [Publisher Full Text](#)
56. Ryan D, Drysdale AJ, Lafourcade C, *et al.*: **Cannabidiol targets mitochondria to regulate intracellular Ca²⁺ levels.** *J Neurosci.* 2009; **29**(7): 2053–2063.
[PubMed Abstract](#) | [Publisher Full Text](#)
57. Devinsky O, Whalley BJ, Di Marzo V: **Erratum to: Cannabinoids in the Treatment of Neurological Disorders.** *Neurotherapeutics.* 2015; **12**(4): 910.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
58. Devinsky O, Whalley BJ, Di Marzo V: **Cannabinoids in the Treatment of Neurological Disorders.** *Neurotherapeutics.* 2015; **12**(4): 689–691.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
59. Friedman D, Devinsky O: **Cannabinoids in the Treatment of Epilepsy.** *N Engl J Med.* 2015; **373**(11): 1048–1058.
[PubMed Abstract](#) | [Publisher Full Text](#)
60. Rosenberg EC, Tsien RW, Whalley BJ, *et al.*: **Cannabinoids and Epilepsy.** *Neurotherapeutics.* 2015; **12**(4): 747–768.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
61. Cilio MR, Thiele EA, Devinsky O: **The case for assessing cannabidiol in epilepsy.** *Epilepsia.* 2014; **55**(6): 787–790.
[PubMed Abstract](#) | [Publisher Full Text](#)
62. Devinsky O, Cilio MR, Cross H, *et al.*: **Cannabidiol: pharmacology and potential therapeutic role in epilepsy and other neuropsychiatric disorders.** *Epilepsia.* 2014; **55**(6): 791–802.
[PubMed Abstract](#) | [Publisher Full Text](#)

Open Peer Review

Current Peer Review Status:

Version 1

Reviewer Report 04 February 2016

<https://doi.org/10.5256/f1000research.8086.r11800>

© 2016 Cooper D. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

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 confirm that I have read this submission and 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 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.

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 patients⁴⁻⁶. 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 3A5⁷. Some of these enzymes have been implicated in the metabolism of the fungal toxins aflatoxin and ochratoxin⁸⁻¹⁰. 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 media⁶⁻⁸. 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 culturable⁹.

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 techniques¹⁰⁻¹⁵.

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 be 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 models⁴⁴⁻⁴⁶. Paxilline is reported to have tremorgenic effects at nanomolar concentrations and is responsible for Ryegrass-staggers disease⁴⁷. 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 interaction⁵⁶. 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 epilepsy⁵⁷⁻⁶², 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.

Reviewer Report 17 December 2015

<https://doi.org/10.5256/f1000research.8086.r11516>

© 2015 McPartland J. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

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 *Kabatella* (*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.

References

1. Cao HX, Schmutzer T, Scholz U, Pecinka A, et al.: Metatranscriptome analysis reveals host-microbiome interactions in traps of carnivorous Genlisea species. *Front Microbiol.* 2015; **6**: 526 [PubMed Abstract](#) | [Publisher Full Text](#)
2. Delmotte N, Knief C, Chaffron S, Innerebner G, et al.: Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. *Proc Natl Acad Sci U S A.* 2009; **106** (38): 16428-33 [PubMed Abstract](#) | [Publisher Full Text](#)
3. Gautam A, Kant M, Thakur Y: Isolation of endophytic fungi from *Cannabis sativa* and study their antifungal potential. *Archives Of Phytopathology And Plant Protection.* 2013; **46** (6): 627-635 [Publisher Full Text](#)
4. Gzebenyuk NV: The occurrence of fungi on hemp stems. *Mikologiya i Fitopatologiya.* 1984; **18** (4): 322-326
5. Kurup VP, Resnick A, Kagen SL, Cohen SH, et al.: Allergenic fungi and actinomycetes in smoking materials and their health implications. *Mycopathologia.* 1983; **82** (1): 61-4 [PubMed Abstract](#)
6. Kusari P, Kusari S, Spiteller M, Kayser O: Endophytic fungi harbored in *Cannabis sativa* L.: diversity and potential as biocontrol agents against host plant-specific phytopathogens. *Fungal Diversity.* 2013; **60** (1): 137-151 [Publisher Full Text](#)
7. Llewellyn GC, O'Rear CE: Examination of fungal growth and aflatoxin production on marihuana. *Mycopathologia.* 1977; **62** (2): 109-12 [PubMed Abstract](#)
8. McPartland JM, Clarke RC, Watson DP: Hemp diseases and pests. *CABI Publishing (Wallingford;UK)* . 2000.
9. Moody MM, Wharton RC, Schnaper N, Schimpff SC: Do water pipes prevent transmission of fungi from contaminated marijuana?. *N Engl J Med.* 1982; **306** (24): 1492-3 [PubMed Abstract](#)
10. Taylor DN, Wachsmuth IK, Shangkuan YH, Schmidt EV, et al.: Salmonellosis associated with marijuana: a multistate outbreak traced by plasmid fingerprinting. *N Engl J Med.* 1982; **306** (21): 1249-53 [PubMed Abstract](#) | [Publisher Full Text](#)
11. Turner TR, James EK, Poole PS: The plant microbiome. *Genome Biol.* 2013; **14** (6): 209 [PubMed](#)

[Abstract](#) | [Publisher Full Text](#)

12. Ungerleider JT, Andrysiak T, Tashkin DP, Gale RP: Contamination of marihuana cigarettes with pathogenic bacteria--possible source of infection in cancer patients. *Cancer Treat Rep*. 1982; **66** (3): 589-91 [PubMed Abstract](#)

13. Vorholt JA: Microbial life in the phyllosphere. *Nat Rev Microbiol*. 2012; **10** (12): 828-40 [PubMed Abstract](#) | [Publisher Full Text](#)

14. Winston ME, Hampton-Marcell J, Zarraonaindia I, Owens SM, et al.: Understanding cultivar-specificity and soil determinants of the cannabis microbiome. *PLoS One*. 2014; **9** (6): e99641 [PubMed Abstract](#) | [Publisher Full Text](#)

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and 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

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.

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 studies¹⁻³. 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 toxin³. 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 citrinum clade¹²) 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.

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.

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™, 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 times⁴⁰. Of the *Penicillium*, only *P. citrinum* has been previously reported to culture with 3M Petri-Film⁴¹. 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*.

fixed

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.

The benefits of publishing with F1000Research:

- Your article is published within days, with no editorial bias
- You can publish traditional articles, null/negative results, case reports, data notes and more
- The peer review process is transparent and collaborative
- Your article is indexed in PubMed after passing peer review
- Dedicated customer support at every stage

For pre-submission enquiries, contact research@f1000.com

F1000Research