No Silver Bullet: Technical barriers to the use of qPCR for the enumeration of microbial contaminants in Cannabis

(Supplementary Materials)

Materials and Methods

Enumeration and DNA extraction from pure culture

For each bacteria and yeast (Table S3), triplicate cultures were grown overnight in tryptic soy broth (TSB) or yeast peptone dextrose broth (YPD), respectively. Spore suspensions of mold cultures (Table S3) were prepared in triplicate as described elsewhere[1]. Before use, bacterial and fungal cultures were standardized to approximately 10^8 and 10^6 CFU/ml, respectively. These standardized cultures were enumerated by spread plate on tryptic soy agar (TSA) and Sabouraud dextrose agar (SDA) respectively for bacteria and fungi. Microscope counts of cultures were used to confirm the absence of an overabundance dead cells which could affect the correlation between plate count and qPCR enumeration. Each standardized culture was decimally diluted in phosphate buffered saline (PBS) up to seven times, and total DNA was extracted from selected dilutions using the MagMAX™ Microbiome Ultra Nucleic Acid Isolation Kit from Applied Biosystems, following the manufacturer's directions.

Enumeration and DNA extraction from cannabis matrix

Triplicate one-gram sub-samples from each flower sample were homogenized in 9 ml of TSB. Each sub-sample was enumerated by standard spread plating (USP general chapter <61>). In parallel, DNA was extracted from each sub-sample using both the MagMAX™ Microbiome

[1] Ricardo Araujo, Acacio G. Rodrigues, and Cidalia Pina-Vaz, "A Fast, Practical and Reproducible Procedure for the Standardization of the Cell Density of an Aspergillus Suspension," *Journal of Medical Microbiology* 53, no. 8 (2004): 783–86, https://doi.org/10.1099/jmm.0.05425-0.

Ultra Nucleic Acid Isolation Kit (Applied Biosystems) and the DNA extraction kit provided by the manufacturer of the commercial qPCR assays assessed in this study. Both kits were used according to the manufacturers' directions.

Amplification with qPCR

All qPCR reactions were performed on an Applied Biosystems Quant-Studio 6 Pro qPCR instrument. For non-commercial assays (Tables S1 & S2), reaction mixtures were prepared using the PowerUp™ SYBR™ Green Master Mix, 500 nM of each primer, and 5 µl of DNA template. Commercial assay reactions were prepared following the manufacturer's directions. Amplification was conducted following the manufacturers' temperature protocols for the respective kit or master mix. Melt curves were prepared for each SYBR™ Green assay to confirm the absence of off-target amplification. For pure culture and negative control assays, qPCR reactions were prepared with DNA extracted using the MAGMAX™ kit. For the cannabis matrix assays, non-commercial assays (Tables S1 & S2) were conducted using DNA extracted with the MAGMAX™ kit, while the commercial assays were conducted using DNA extracted using the DNA extraction kit provided by the assay manufacturer.

Data Analysis

Raw qPCR data was collected using the Design & Analysis Software (v2.4; Applied Biosystems). Collected data was analyzed using R software (v3.6.3; R Foundation for Statistical Computing, [https://www.R-project.org/].

Supplementary Tables

Table S1. Primer details for each total aerobic microbial count assay

Assay	Target	Length (bp)	Primer	Sequence (5' – 3')[a]	Reference
V4	16S rRNA	275	V4_F V4_R	GTGCCAGC**M**GCCGCGGTAA GGACTAC**NV**GGGT**W**TCTAAT	[2]
V3V4	16S rRNA	466	V3V4_F V3V4_R	TCCTACGGGAGGCAGCAGT GGACTACCAGGGTATCTAATCCTGTT	[3]
rpoB	*rpoB*	430	rpoB_F rpoB_R	GG**Y**T**WY**GAAGT**N**CG**H**GACGT**D**CA TGACG**Y**TGCATGTT**B**G**MR**CCCAT**M**A	[4]
gyrB	*gyrB*	280	gyrB_F gyrB_R	**M**GN**N**CC**N**G**SN**ATGTA**Y**AT**H**GG C**N**CC**R**TGN**A****RD**CC**D**CC**N**GA	[5]

[a]Degenerate bases are highlighted in bold

[2] J. Gregory Caporaso et al., "Global Patterns of 16S RRNA Diversity at a Depth of Millions of Sequences per Sample," *Proceedings of the National Academy of Sciences of the United States of America* 108, no. SUPPL. 1 (2011): 4516–22, https://doi.org/10.1073/pnas.1000080107.

[3] Mangala A. Nadkarni et al., "Determination of Bacterial Load by Real-Time PCR Using a Broad-Range (Universal) Probe and Primers Set," *Microbiology* 148, no. 1 (2002): 257–66, https://doi.org/10.1099/00221287-148-1-257.

[4] Jean Claude Ogier et al., "RpoB, a Promising Marker for Analyzing the Diversity of Bacterial Communities by Amplicon Sequencing," *BMC Microbiology* 19, no. 1 (2019): 1–16, https://doi.org/10.1186/s12866-019-1546-z.

[5] Simon Poirier et al., "Deciphering Intra-Species Bacterial Diversity of Meat and Seafood Spoilage Microbiota Using GyrB Amplicon Sequencing: A Comparative Analysis with 16S RDNA V3-V4 Amplicon Sequencing," *PLoS ONE* 13, no. 9 (2018): 1–26, https://doi.org/10.1371/journal.pone.0204629.

Table S2. Primer details for each total yeast and mold count assay

Assay	Target	Length (bp)	Primer	Sequence (5' – 3')[a]	Reference
ITS1	ITS[b]	200-250	ITS1_F ITS1_R	TCCGTAGGTGAACCTGCGG GCTGCGTTCTTCATCGATGC	[6]
ITS2	ITS	450-550	ITS2_F ITS2_R	ATCGATGAAGAACGCAG GGATTCTCACCCTCTATGAC	[7]
TEF1α	*tef1A*	600	TEF_F TEF_R	GA**Y**TTCATCAAGAACATGAT GACGTTGAA**D**CC**R**AC**R**TTGTC	[8]
LNS2	*LNS2*	400	LNS_F LNS_R	GGCCATGTGCTGAACATGATCGG**H**CG**W**GA**Y**TGGAC CGGTTGCC**R**AA**K**CC**R**GCATAGAA**K**GG	[9]

[a]Degenerate bases are highlighted in bold
[b]Internal transcribed spacer

[6] Nicholas A. Bokulich and David A. Mills, "Improved Selection of Internal Transcribed Spacer-Specific Primers Enables Quantitative, Ultra-High-Throughput Profiling of Fungal Communities," *Applied and Environmental Microbiology* 79, no. 8 (2013): 2519–26, https://doi.org/10.1128/AEM.03870-12.
[7] Bokulich and Mills.
[8] J. B. Stielow et al., "One Fungus, Which Genes? Development and Assessment of Universal Primers for Potential Secondary Fungal DNA Barcodes," *Persoonia: Molecular Phylogeny and Evolution of Fungi* 35, no. 1 (2015): 242–63, https://doi.org/10.3767/003158515X689135.
[9] Stielow et al.

Table S3. Microorganisms used in this study

Species	Strain	Assay	Sub-group
Bacillus subtilis	NCTC 10400	TAC	Gram-positive
Staphylococcus aureus	NCTC 10788	TAC	Gram-positive
Pseudomonas aeruginosa	NCTC 12924	TAC	Gram-negative
Escherichia coli	NCTC 9001	TAC	Gram-negative
Aspergillus brasiliensis	NCPF 2275	TYM	Mold
Penicillium chrysogenum	ATCC 10002	TYM	Mold
Candida albicans	NCPF 3179	TYM	Yeast
Saccharomyces cerevisiae	CEN.PK2-1D	TYM	Yeast