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

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Key Points

- Variations in target gene copy number and cells per colony-forming unit can result in inter-organism discrepancies by qPCR-based enumeration.
- Differences in DNA quality between extraction kits can affect the quality and results of qPCR enumeration assays.
- The ubiquitous presence of DNA contamination in laboratory reagents prevents accurate enumeration in low biomass samples.
- The presence of DNA from dead microbes in dried cannabis results in overestimation of microbial load by qPCR relative to culture-based methods.

Introduction

With the legalization of cannabis in Canada and other jurisdictions around the globe, strict testing requirements are being mandated to ensure product quality and safety is of the highest standard. Such cannabis must undergo extensive chemical and microbiological analysis to determine potency, and to identify and quantify any impurities or contaminants. For microbial analysis, required tests often include determination of the total aerobic microbial count (TAC) and the total yeast and mold counts (TYM)[1]. While United States Pharmacopeia (USP) and European Pharmacopeia (Ph. Eur.) guidance recommends culture-based plating methods, it can take up to seven days to acquire results (Figure 1).

Quantitative Polymerase Chain Reaction (qPCR) has been gaining attention in the cannabis industry as a faster alternative to culture-based testing (Figure 1). In qPCR, a gene of interest (DNA region) is copied/amplified in a cyclical reaction using a pair of DNA primers targeted to that gene, along with a fluorescent dye that binds the newly formed DNA. The increasing number of target gene copies (amplicons) are monitored through an increase in fluorescence signal that is proportional to the number of amplicons present. The amplification cycle number (Cq value) where the fluorescence increases beyond a specified threshold is log-linearly correlated to the starting concentration of the target, allowing quantitation of that gene. Although qPCR methods require technical expertise, specialized equipment, and expensive reagents, results can be generated in as little as a few hours.

While qPCR has been used to detect or enumerate individual microbial taxa in food[2] and water[3] safety testing, it has not been third party validated to USP, Ph. Eur, AOAC, or other applicable standards for the enumeration of complex microbial communities like those in Cannabis, which may contain hundreds of different microbial species[4]. For qPCR to accurately quantify the microbes in a multi-species community, the method must (1) equally target all members of the group

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[1] Sarma et al. *Journal of Natural Products* 83, no. 4: 1334–51, https://doi.org/10.1021/acs.jnatprod.9b01200.

[2] *Comprehensive Reviews in Food Science and Food Safety* 13, no. 4 (2014): 551–77, https://doi.org/10.1111/1541-4337.12072.

[3] Deshmukh et al. *MicrobiologyOpen* 5, no. 6 (2016): 901–22, https://doi.org/10.1002/mbo3.383.

[4] Comeau et al. *Frontiers in Microbiology* 11, March (2020): 1–14 https://doi.org/10.3389/fmicb.2020.00491.

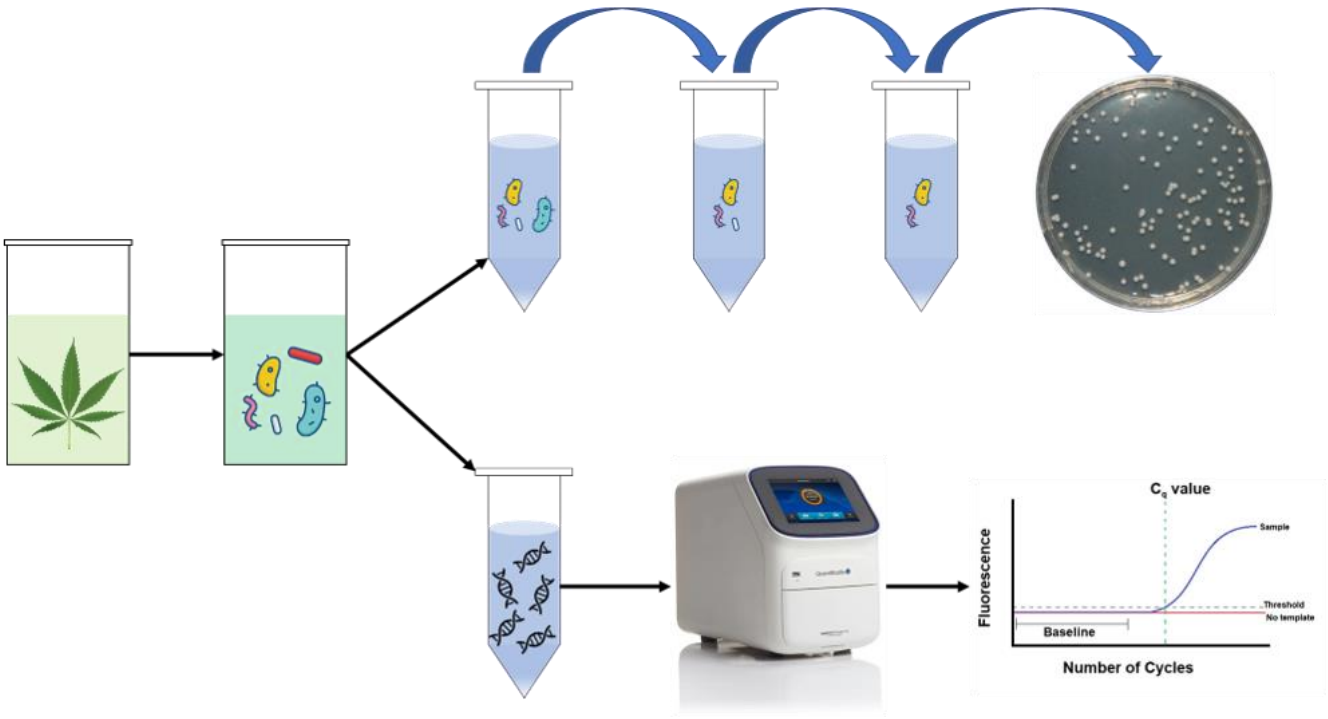

Figure 1. Typical Sample preparation steps for USP/EP Herbal Limits by culture-based plating (top) and by qPCR (bottom)

being quantified (*e.g.*, all bacteria or all fungi), (2) be sensitive enough to enumerate microbes at or below regulatory limits, and (3) correlate with results from standard methods (*i.e.*, culture-based plating).

In this report, we assessed the suitability of qPCR for the enumeration of TAC and TYM in dried cannabis. Specifically, we (i) compared a variety of primer pairs and commercial assays for microbial enumeration, (ii) assessed the influence of DNA contamination in qPCR reagents on the lower limits of detection, and (iii) compared the results of qPCR enumeration to standard plate counts in dried cannabis and hemp samples. Despite some success with single, pure-culture organisms, our results identified several concerns which must be addressed before qPCR can be considered a reliable tool for microbial enumeration in the cannabis industry.

Comparison of primer sets for total microbial enumeration in Cannabis

The most important factor in a qPCR microbial enumeration assay is primer pair selection. The primers must completely and equally target all members of the group being enumerated (*i.e.*, total bacteria or total yeast and mold). The most common "universal" primer sets target the 16S ribosomal RNA (16S rRNA) in bacteria or the ribosomal internally transcribed spacer (ITS) in fungi.

Our first objective was to compare a variety of primer pairs (assays) designed to target all bacteria (TAC) or all fungi (TYM). These assays are outlined in Tables S1 and S2 in the supplementary materials. We also included commercially available assays for qPCR enumeration of total aerobic count (C-TAC) and total yeast and mold (C-TYM). All TAC and TYM assays were tested with the organisms listed in Table S3. Triplicate cultures of each bacterium (approx. 10^8 CFU/ml) and fungus (approx. 10^6 CFU/ml) were decimally diluted 4 times, and each dilution was enumerated in parallel by standard plate count, and by qPCR with each relevant assay. For consistency, all qPCR assays for each dilution were conducted with the same extracted DNA. Pure cultures were used to avoid possible variations and effects from the dried cannabis matrix. For a detailed

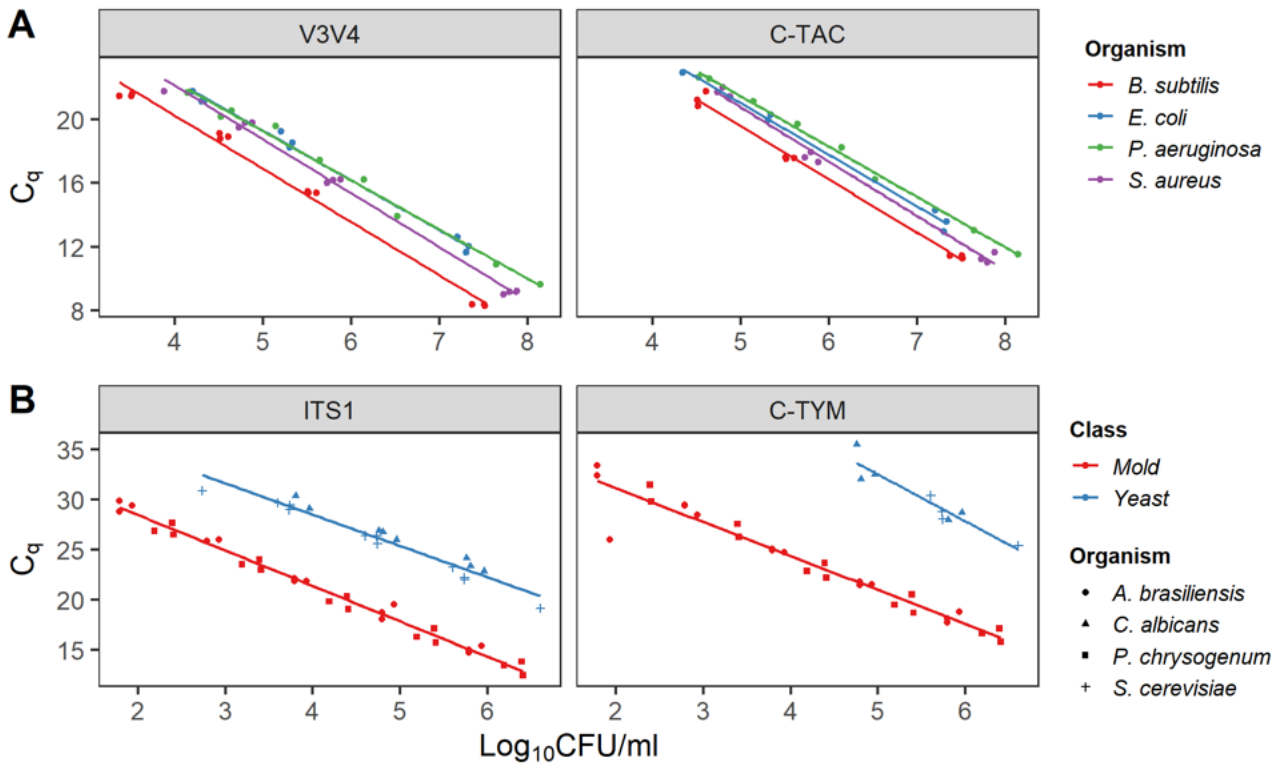

Figure 2. Correlation curves between standard plate count (log10 CFU/ml) and qPCR (C_q) for four bacteria (A) and four fungi (B) using assays designed to enumerate total aerobic bacteria (V3V4 & C-TAC) and total yeast and mold (ITS1 & C-TYM).

description of methods, please see the supplementary materials[5].

For brevity, only the best performing assays for TAC (V3V4 region of the 16S rRNA) and TYM (ITS1) are included here with the two commercial assays for comparison. For each bacterium individually, a strong correlation ($r > 0.99$) was observed between standard plate counts and C_q for both the V3V4 and C-TAC assays. These results imply that the assays are functioning properly, and that the generated data is reliable.

Problems arise, however, when comparing results between the different species of bacteria (Figure 2A). Both the V3V4 and C-TAC assays yielded a greater than 0.5 log CFU (approx. 70%) difference between the CFU-to-C_q response curves of *B. subtilis* and other species. This means the assays would estimate different microbial loads depending on the composition of bacteria in the sample. For example, a C-TAC assay C_q value of 21 would suggest 37,000 CFU/g of *B. subtilis,* but 138,000 CFU/g of *P. aeruginosa*; an important distinction when compendial guidance recommends no more than 100,000 CFU/g TAC for cannabis inflorescence[6].

TYM enumeration primers were even less reliable. While the ITS assay had strong correlations between C_q and plate count for each fungal species ($r \geq 0.99$), the commercial C-TYM assay correlations were weaker, especially for *S. cerevisiae* ($r = 0.94$) and *C. albicans* ($r = 0.91$). These results are of consequence since they are below the acceptance criteria described in USP <1223>, which requires a correlation above 0.95. Of greater consequence is the large difference in response between the yeast and mold species (Figure 1B). The ITS1 assay shows an approximately 100-fold difference in CFU counts at any given C_q value, and this same difference can be as high as 1,000-fold in the C-TYM assay.

[5] Please email us at testing@willowbio.com for Supplemental Materials.

[6] Sarma et al. *J. Nat. Prod.* 83, no. 4: 1334–51

The observed inter-species variation for both TAC and TYM indicates that qPCR enumeration is not reliable across groups of microbial species, including commercial kits currently available on the market. One explanation for these discrepancies is variation in the copy number of the target gene. Variations in the number of copies of the 16S rRNA[7] and ITS[8] DNA regions have been observed across bacterial and fungal taxa, respectively. Efforts have been made to identify other, single-copy, universal DNA targets for bacteria and fungi, but none of those we tested (Tables S1 and S2) provided acceptable results across our target organisms. A second explanation is the distinction between CFU and individual cells. Bacteria often exist in clumps and chains of varying sizes which, due to proximity, will only result in a single CFU[9]. For these reasons, laboratories considering the use of qPCR-based methods for microbial enumeration should conduct their own, in-house validations using a variety or target organisms before accepting these assays for routine use.

Background signal and limit of detection

In qPCR enumeration assays, the limit of detection (LOD) is usually defined as the minimum number of cells required for successful amplification. However, in total microbial enumeration assays (*e.g.*, TAC and TYM), any microbial DNA contamination in assay reagents is detected and enumerated, affecting LOD, and preventing lower detection and quantification limits. For example, additional dilutions of the bacteria samples using the V3V4 assay level off at a C_q between 21 and 22, representing the level of bacterial DNA in the reagents (Figure 3A).

Microbial DNA contamination is ubiquitous, but highly variable across laboratory reagents and DNA extraction kits[10]. Importantly, this contaminating DNA cannot be removed through autoclaving. As shown in Figure 3B, when we tested two different brands of phosphate buffered saline (PBS) – the diluent used for our experiments – with the V3V4 primer set, we observed substantial but very different levels of bacterial DNA contamination (>6-cycle difference in C_q value). Contamination was even observed for the qPCR reagents (*i.e.*, no-template control) with the V3V4 primer set. Later amplification of the PBS diluents was observed for the ITS1 primer set, implying that most, but not all, contaminating DNA is from bacteria.

To assess how DNA contamination in reagents affects the LOD of qPCR microbial enumeration assays, we prepared low concentration correlation curves for the V3V4 and ITS1 assays using a molecular grade PBS diluent (Figure 3 C&D). For the V3V4 assay, the LOD ranged from 2.8 log (approx. 630) CFU/ml to 3.5 log (approx. 3,200) CFU/ml for *B. subtilis* and *P. aeruginosa,* respectively. The ITS1 mold sample had an LOD of 1.5 log (approx. 32) CFU/ml, but the LOD was substantially higher in yeast samples, having a similar LOD to the V3V4 assay (3.3 log CFU/ml). These limits are of consequence since the recommended USP limits for TAC and TYM in inhaled products are 10^2 and 10^1 CFU/g, respectively[11].

Comparing qPCR to standard plate counts in Cannabis

According to USP <1223> *Validation of Alternative Microbiological Procedures*, an alternative quantitative microbiological method like qPCR should produce results that correlate closely (r >0.95) with compendial plating methods for a given sample. To investigate the reliability of the above-described qPCR assays to enumerate TAC and TYM in dried cannabis, we compared standard plate counts

[7] Větrovský and Baldrian. *PLoS ONE* 8, no. 2 (2013): 1–10, https://doi.org/10.1371/journal.pone.0057923.
[8] Lofgren et al. *Molecular Ecology* 28, no. 4 (2019): 721–30, https://doi.org/10.1111/mec.14995.
[9] Auty et al. *Applied and Environmental Microbiology* 67, no. 1 (2001): 420–25, https://doi.org/10.1128/AEM.67.1.420-425.2001.
[10] Salter et al. *BMC Biology* 12, no. 1 (2014): 1–12, https://doi.org/10.1186/s12915-014-0087-z.
[11] Sarma et al. *J. Nat. Prod.* 83, no. 4: 1334–51,

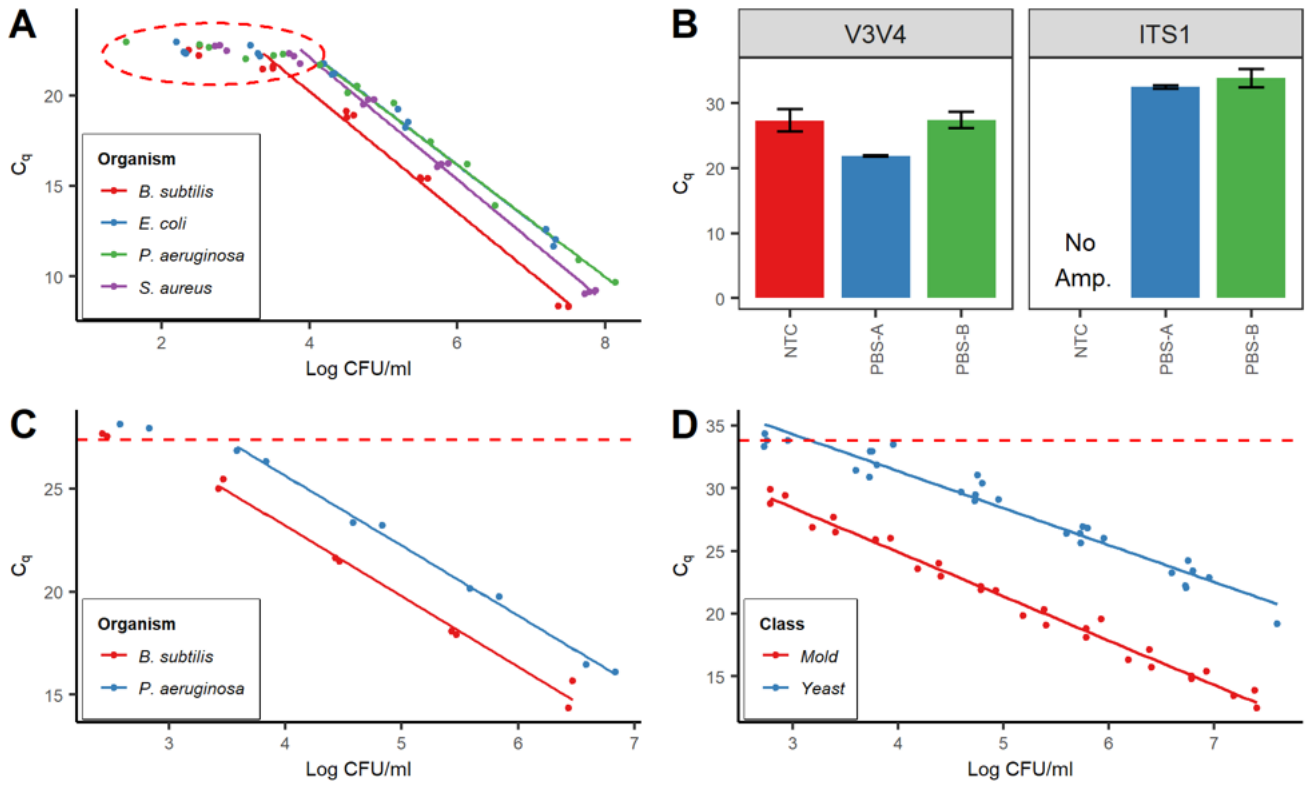

Figure 3. Investigation of DNA contamination in laboratory reagents and their effect on the limit of detection (LOD) in qPCR assays. (A) correlation curves (C_q vs log10 CFU/ml) for dilutions of four bacteria show a plateau (red circle) at the level of background DNA in the phosphate buffered saline (PBS) diluent. (B) Resulting C_q values for two different brands of PBS after DNA extraction, and a no-template control (NTC) qPCR reaction. (C & D) The LOD for two bacteria using the V3V4 assay (C), and yeast and mold using the ITS1 assay (D). The red dashed line represents the mean C_q for a molecular grade PBS used as a diluent for the microbial samples.

to qPCR results in triplicate from four dried cannabis samples and two hemp samples. Two of the dried cannabis samples were irradiated before collection. The V3V4 and ITS1 assays were tested with DNA extracted using our in-house protocol while the two commercial assays were tested with DNA extracted using the manufacturer's proprietary DNA extraction kit.

For all four qPCR assays, no suitable correlation between the predicted and actual TAC and TYM concentrations was observed (Figure 4); however, the V3V4 and ITS1 assays both outperformed their commercial counterparts. Of additional concern, the correlation between plate count and C_q value was reversed in both commercial assays, showing higher DNA concentrations in samples with lower plate counts.

There are a couple possible explanations for the lack of correlation between qPCR and standard plate counts. The first is the possible presence of non-viable DNA from dead cells. The drying and/or irradiation process in cannabis will inactivate many, if not all the microbial cells, but the DNA is still present and detectable by qPCR, resulting in an overestimation of living microbes. While there are laboratory methods that may remove or prevent non-viable DNA from being amplified, these methods are far from perfect[12]. The second possibility is the presence of PCR inhibitors. Plants are known to carry compounds that interfere with PCR

[12]Emerson et al. *Microbiome* 5, no. 1 (2017): 86, https://doi.org/10.1186/s40168-017-0285-3.

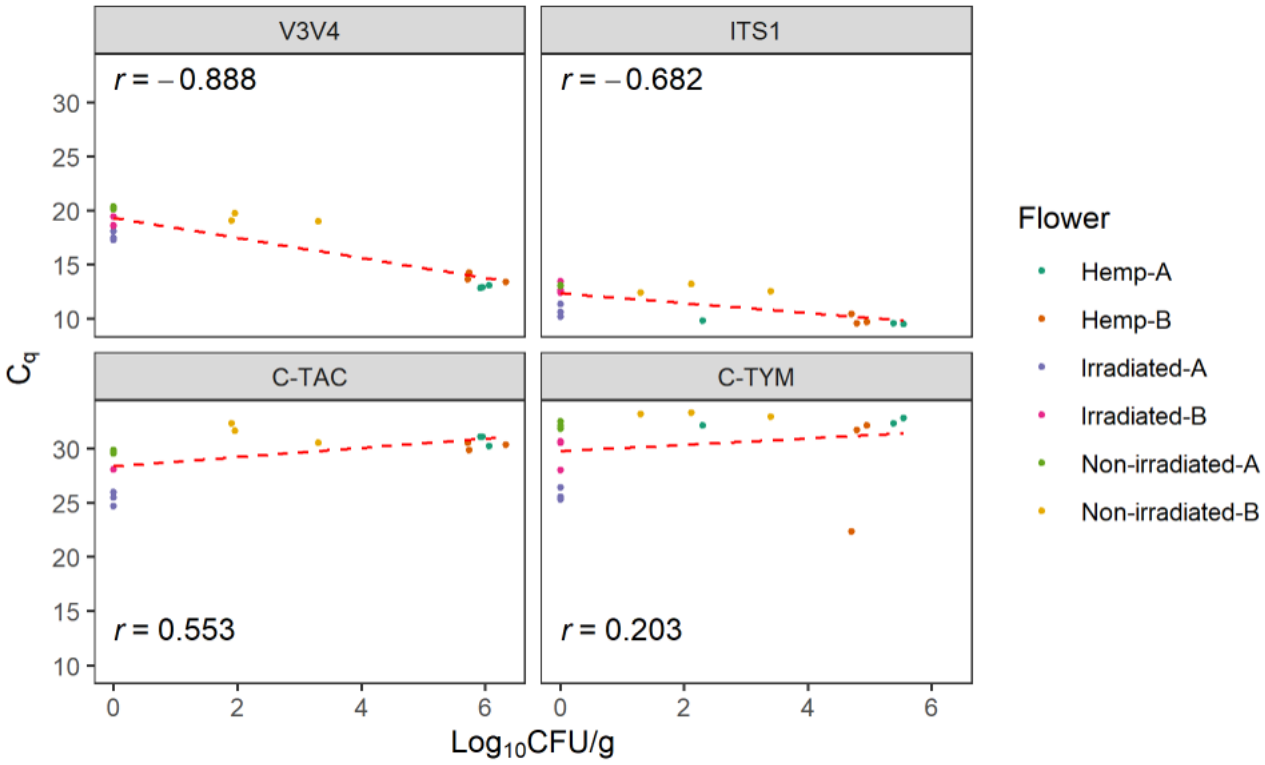

Figure 4. Correlation curves and correlation coefficients (r) between standard plate count (log10 CFU/ml) and qPCR (Cq) for enumeration of total aerobic bacteria (V3V4 & C-TAC) or total yeast and mold (ITS1 & C-TYM) in triplicate aliquots from four cannabis (two irradiated and two non-irradiated) and two hemp samples.

amplification[13]. If these compounds were not completely removed during DNA isolation process, they can lead to later amplification, and an underestimation of total microbial load. Therefore, DNA extraction protocols needs to be thoroughly validated along with the qPCR assay. Further, matrix validations performed by inoculating with purified DNA should not be considered reliable.

Summary and conclusions

At first glance, qPCR appears to provide a rapid alternative to culture-based microbial methods, but a variety of technical issues make this technology unreliable for TAC and TYM enumeration in cannabis. Variation in qPCR response between microbial species, most notably in TYM enumeration, make accurate conversion of C_q to CFU extremely difficult, if not impossible. Additionally, the presence of contaminating DNA in reagents can affect the ability to enumerate at lower regulatory limits. Finally, matrix effects such as the presence of dead DNA and/or PCR inhibitors can interfere with accurate enumeration *in situ*. While we agree that qPCR is a powerful tool for the detection of specific microorganisms in cannabis (*e.g.*, *E. coli* or *Aspergillus*), we would not recommend using qPCR in place of traditional culture-based methods for TAC and TYM. Critical research addressing the issues outlined here is necessary before qPCR should be accepted for routine microbial enumeration in Cannabis.

[13]C. Schrader et al. *Journal of Applied Microbiology* 113, no. 5 (2012): 1014–26, https://doi.org/10.1111/j.1365-2672.2012.05384.x.