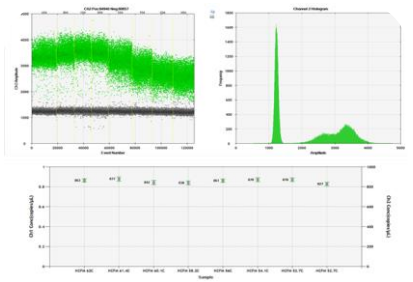
CONFIRMATION OF PROBIOTIC BLEND UNIFORMITY WITH DROPLET DIGITAL PCR

Meeting: USP Virtual Symposium: Emerging technologies in probiotic, live biotherapeutic product and microbiome analysis.

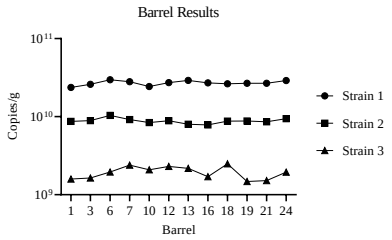
Presenter: Anthony Kiefer

AGENDA

Overview of ddPCR and v-ddPCR.



Current and potential uses of ddPCR at IFF.



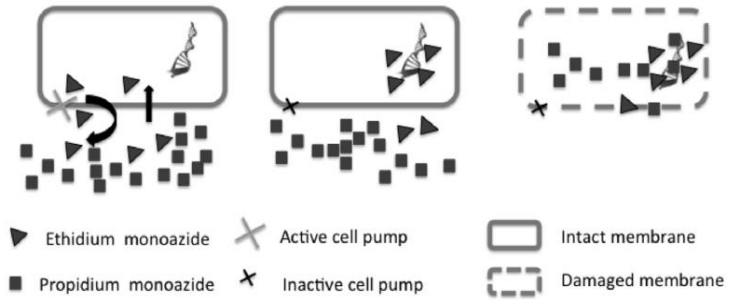
Case study: Confirmation of Probiotic Blend Uniformity with Droplet Digital PCR.

Thank you and references.

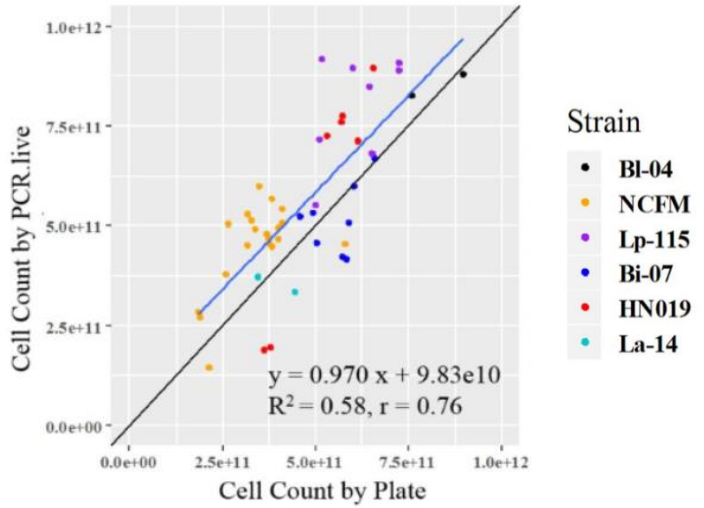
Thoughts, Comments, Questions.

DROPLET DIGITAL PCR (DDPCR) OVERVIEW

- ddPCR represents the latest development in DNA quantification technology.
- ddPCR partitions a sample into oil droplets and uses a Poisson distribution to extrapolate absolute counts from the number of partitions showing amplification of single-copy gene target (Hindson et al., 2011).
- Viability ddPCR (v-ddPCR) using a combination of PMA and EMA (viability dyes) was shown to enumerate several commercial probiotic strains with high correlation to plate counts (Hansen et al., 2020).
- V-ddPCR offers improvements to agar plate counting methods including generating faster (4–8 vs. 24–72 h) and more accurate (1–3% vs. 12–20% CV) results (Hansen et al., 2020).
- V-ddPCR uses specific DNA targets to enumerate single strains of bacteria in multi-strain products, while plate count is only capable of genus level or species level distinction.
- V-ddPCR offers improvements over quantitative PCR (qPCR) such as less sensitivity to inhibitors (Huggett et al., 2013), lower limit of detection (Qian et al., 2016) and does not require a standard curve (Hindson et al., 2011).



(Codony et al., 2015)



(Hansen et al., 2020)

(Bio-Rad, 2022)

PLATE COUNT AND V-ddPCR METHOD SUMMARY

Probiotic Sample

Rehydrate and Dilute

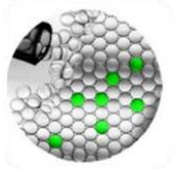
Viability Treatment

Cell Lysis

Sample + PCR Reagents

Droplet Formation

Thermal Cycling



Droplet Reading

Sample plating

Agar Pouring

Incubation

Colony Counting

CURRENT AND FUTURE USES OF DDPCR AND V-DDPCR AT IFF.

- **Current uses are for R&D purposes only.**

- **Strain specific identification in single- and multi-strain products.**

- **Enumeration of single strains in multi-strain matrices.**
  - Multi-strain dietary supplements.
  - (Current/Future) Clinical and animal trial samples (investigative product, fecal, nasal, oral, etc.).
  - (Future) Cultured foods (yogurt, sausage, kombucha, etc.).

- **(Future) Reverse transcriptase ddPCR (rt-ddPCR) to measure gene expression.**

- **(Future) Enumeration of new probiotic genera and postbiotics.**

CONFIRMATION OF PROBIOTIC BLEND UNIFORMITY WITH DROPLET DIGITAL PCR

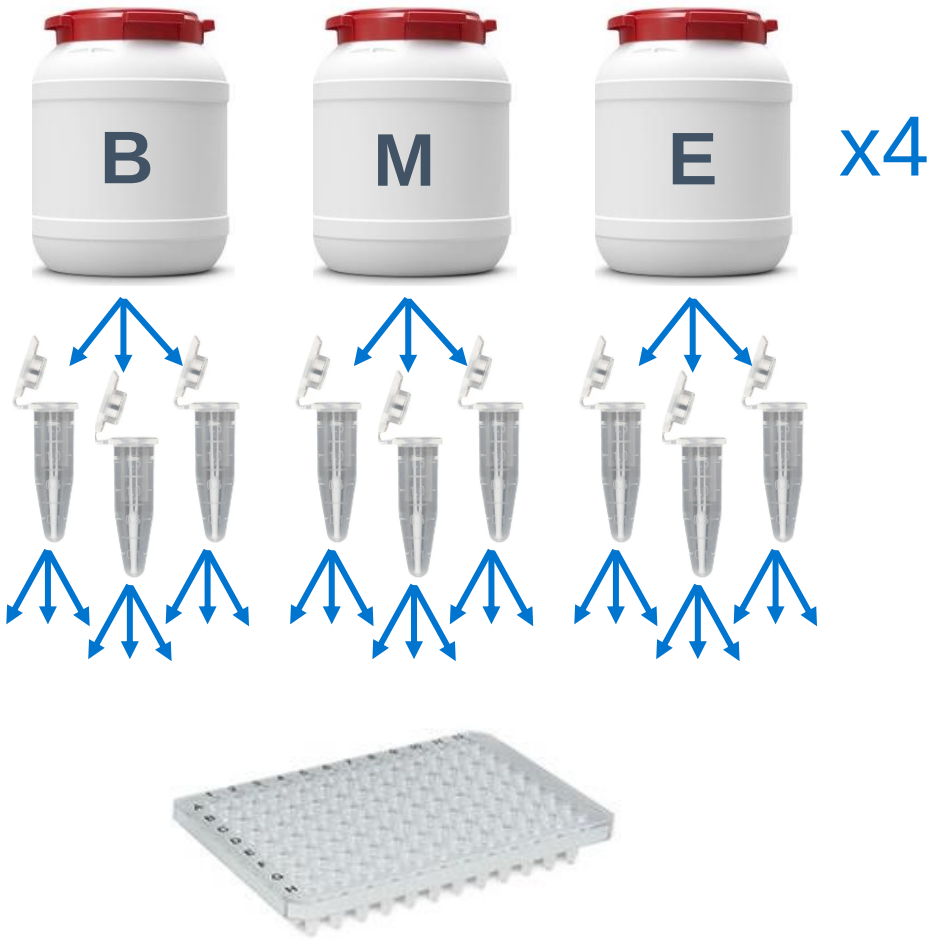
- **Hopper ready blends (HRB) are a common product offered by IFF. HRBs are an intermediate bulk powder which contain all the ingredients needed to make a finished product such as capsules, sachets, tablets, etc.**
- **A single lot of an HRB often contains several 20 kg or 50 kg barrels.**
- **When processing (encapsulating, tableting, sacheting, etc.) the number of barrels used in a run (dump) is dependent on the capacity of the equipment.**
- **A single lot of a finished good may be created from several runs (dumps) using the same lot of HRB.**
- **Question: How do we ensure the lot is uniform between dumps?**

Single lot of HRB (4 barrels)

Multiple dumps (2 barrels per dump)

Single lot of finished product

STUDY DESIGN



- HRB lot consists of 24 barrels.
  - Four separate dumps needed
  - Three barrels sampled per dump (B, M, E)
  - 12 barrels sampled total
- Three strains analyzed based on inclusion rate.
  - Strain 1 (high), Strain 2 (mid), Strain 3 (low)
- Enumerated by v- ddPCR in triplicate.

STATISTICAL ANALYSIS

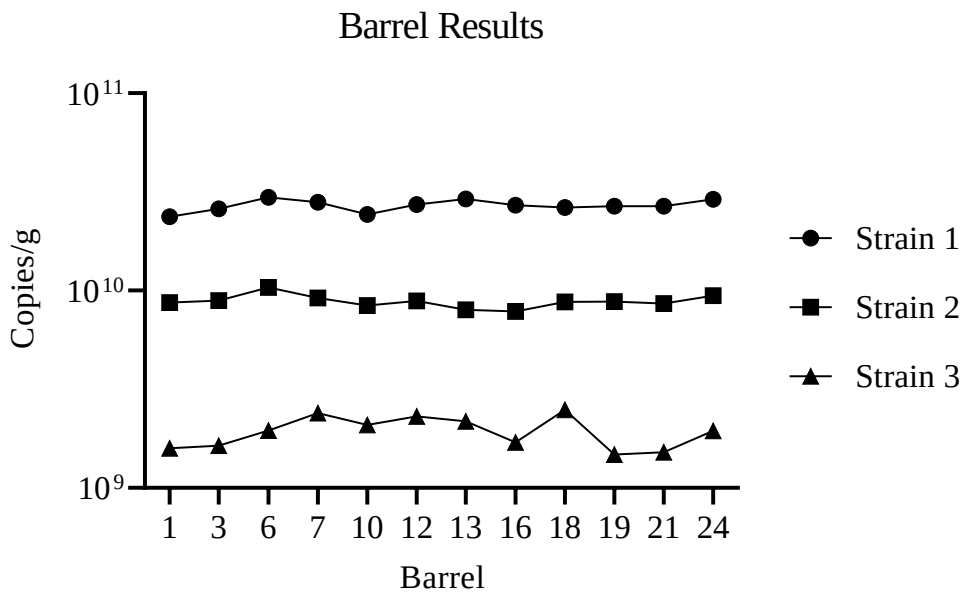


Figure 1: Summary chart of v-ddPCR enumeration data for all barrels.

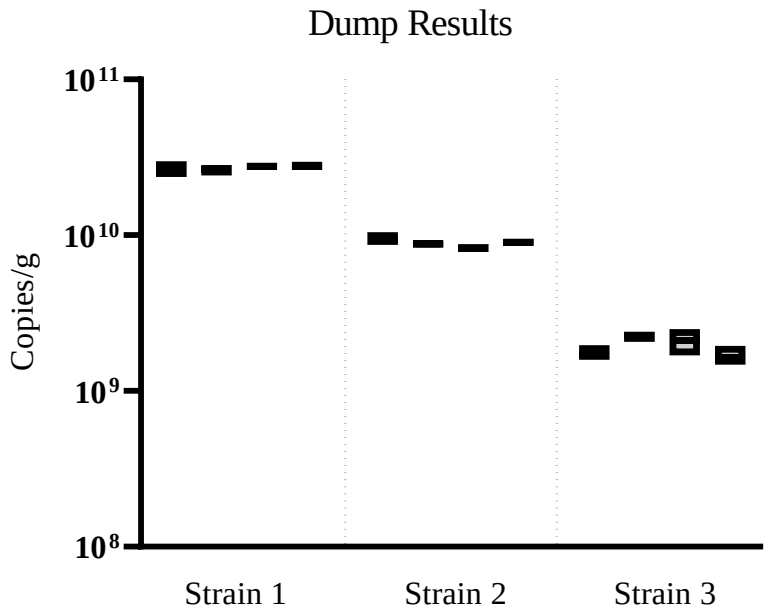


Figure 2: Box plot summarizing average v-ddPCR enumeration data for each strain analyzed, by dump. Dumps 1-4 from left to right.

STATISTICAL ANALYSIS CONTINUED

Strain	RSD (%) entire lot	RSD (%) between dumps	ANOVA (P) between dumps	Significance
Strain 1	6.99%	2.22%	0.8487	ns
Strain 2	7.38%	5.34%	0.221	ns
Strain 3	18.11%	15.32%	0.0635	ns

Table 1: Coefficeint of variation (CV), as reported in %RSD, was calculated for all barrels to determine the uniformity of the entire HRB lot (RSD (%) entire lot). CV was also calculated for the average %RSD of each dump to account for potential differences between dumps (RSD (%) between dumps). Lastly, one-way ANOVA was performed to determine if statistical differences existed between dumps. Results were reported as P-values and a designation of significant (s) or not significant (ns).

CONCLUSIONS

- **v-ddPCR was chosen as the method of analysis in this study for its low CV (desired CV (uniformity) is within the CV of PC method) and its ability to enumerate single strains in a multi-strain probiotic product (PC only capable of total cell count).**

- **Twelve barrels, across 4 dumps (3 barrels per dump), were tested for uniformity. Three strains at high, medium and low inclusion rate (Strain 1, Strain 2, Strain 3 respectively) were chosen for enumeration and subsequent analysis.**

- **Low CV (%RSD) was observed for two of the three strains analyzed (Strain 1 = 6.99%, Strain 2 = 7.38%).**

- **Slightly higher CV (%RSD) was observed for Strain 3 (18.11%) which was the strain with the lowest inclusion rate.**
  - CV was lower (15.32%), near desired upper limit (15%), when comparing dumps rather than individual barrels. ANOVA was not able to detect any significant differences between dumps.

- **No statistical differences between dumps as determined by one-way ANOVA ($P \geq 0.05$).**

SPECIAL THANK YOU TO THE IFF DIGIDROP TEAM!

THOUGHTS, COMMENTS, QUESTIONS.

Thank you!

REFERENCES

- Bio-Rad (2022). Droplet Digital PCR and Technology. Available at: https://www.bio-rad.com/en-us/life-science/digital-pcr?ID=M9HE2R15&WT_knsh_id=_kenshoo_clickid_&WT_mc_id=170125000809&WT_srch=1&gclid=EAIaIQobChMI6t-rpfTF9gIVN21vBB2uVgI4EAAYAyAAEgICUvD_BwE. (accessed March 14, 2022).
- Codony, F., Agustí, G., and Allué-Guardia, A. (2015). Cell membrane integrity and distinguishing between metabolically active and inactive cells as a means of improving viability PCR. Molecular and Cellular Probes 29, 190–192. doi:10.1016/j.mcp.2015.03.003.
- Hansen, S. J. Z., Tang, P., Kiefer, A., Galles, K., Wong, C., and Morovic, W. (2020). Droplet Digital PCR Is an Improved Alternative Method for High-Quality Enumeration of Viable Probiotic Strains. Front. Microbiol. 10. doi:10.3389/fmicb.2019.03025.
- Hindson, B. J., Ness, K. D., Masquelier, D. A., Belgrader, P., Heredia, N. J., Makarewicz, A. J., et al. (2011).
- Huggett, J. F., Foy, C. A., Benes, V., Emslie, K., Garson, J. A., Haynes, R., et al. (2013). The Digital MIQE Guidelines: Minimum Information for Publication of Quantitative Digital PCR Experiments. Clinical Chemistry 59, 892–902. doi: 10.1373/clinchem.2013.206375.
- Qian, L., Song, H., and Cai, W. (2016). Determination of Bifidobacterium and Lactobacillus in breast milk of healthy women by digital PCR. Beneficial Microbes 7, 559–569. doi:10.3920/BM2015.0195.