Optimizing gDNA purification and qPCR techniques for specific detection and enumeration of probiotic bacteria

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Background

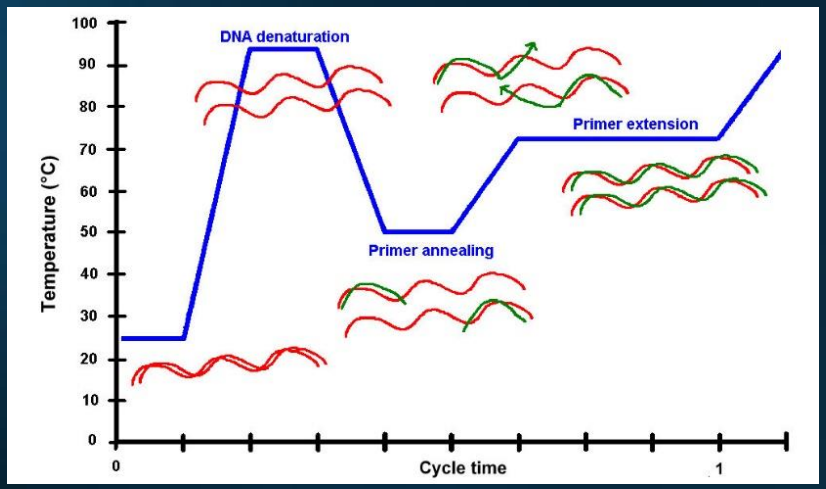
- Probiotics are live microorganisms which are thought to provide health benefits.
- Probiotic products are labeled with the number of cells present at the time of manufacture.

Objective

- Develop an assay which can be used to quantify the number of specific lactic acid bacteria present in specific probiotic products.
  - This process involves isolating genomic DNA (gDNA) from the samples and then running the DNA through a quantitative polymerase chain reaction (qPCR).

Key Terms

- gDNA – genomic DNA, the DNA from the genome of an organism.
- PCR – polymerase chain reaction, amplifies a specific short target sequence of DNA, doubling it every time.

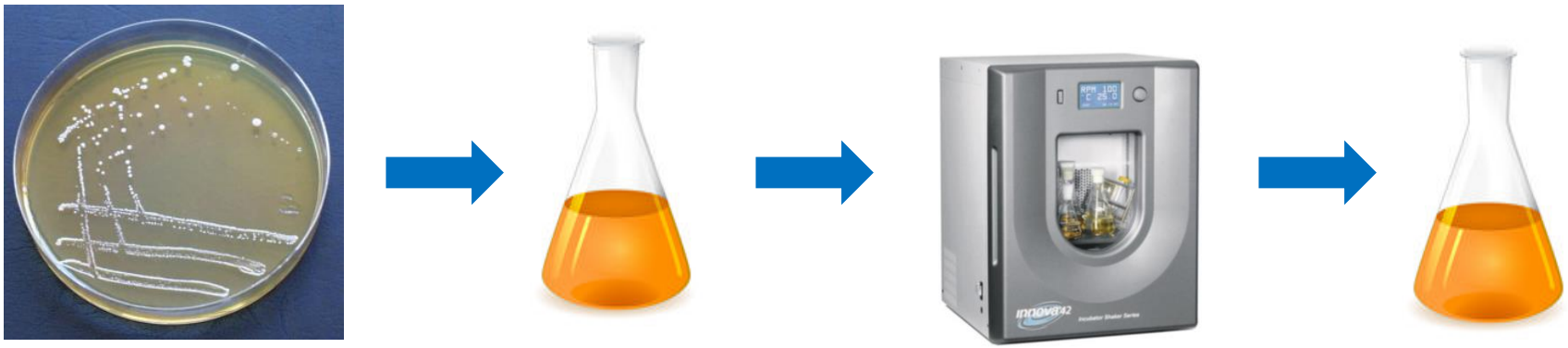


Key Terms

- qPCR – quantitative polymerase chain reaction
  - The DNA amplification produces fluorescence
  - Amount of fluorescence is proportional to amount of amplified DNA.
  - Allows researchers to monitor the amplification of a targeted DNA sequence in real time as the reaction is taking place.

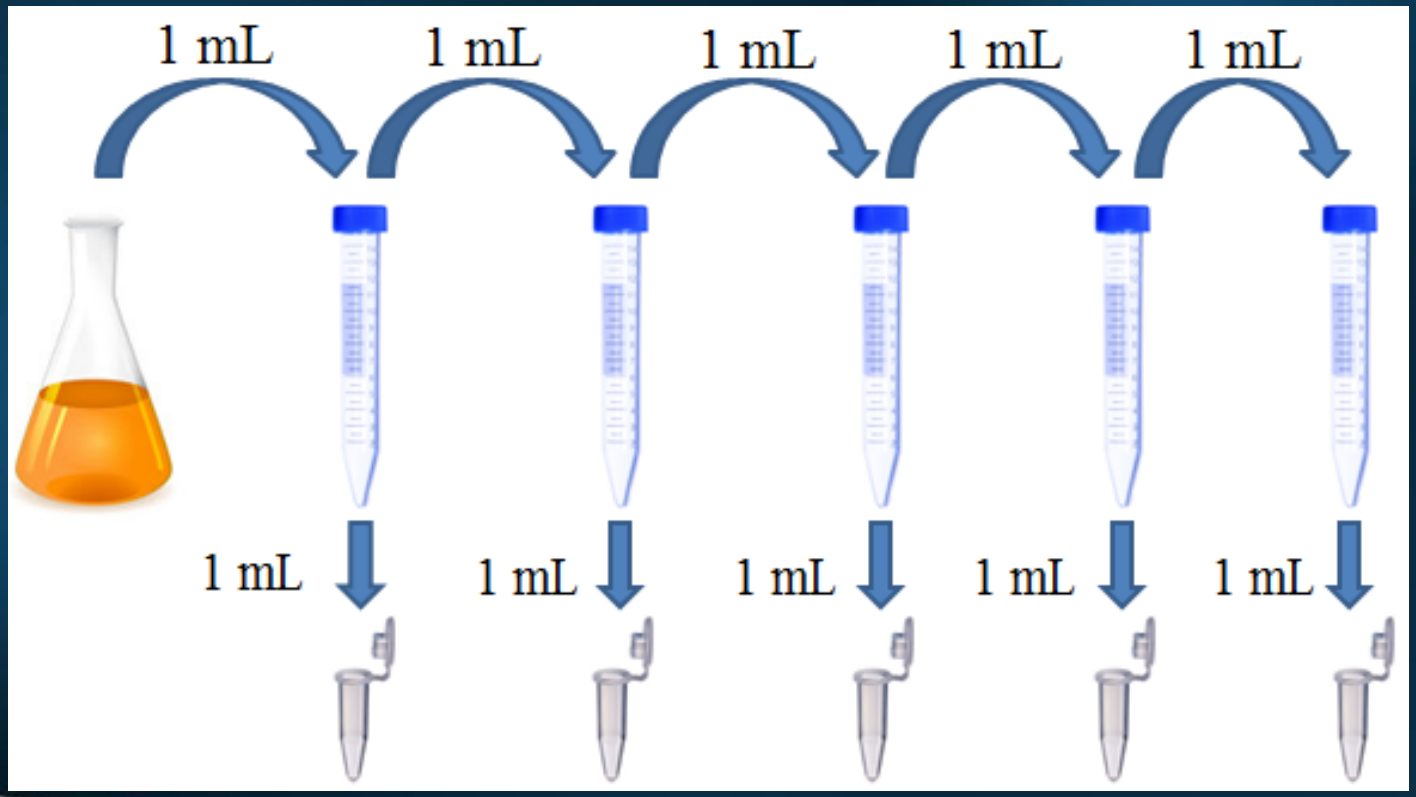
Methods

1. Grow a 25 mL overnight culture of bacteria in MRS at 35 °C.



Methods

2. Spike 10% dextrose with cells and conduct serial dilution: 1×10^9 to 1×10^5

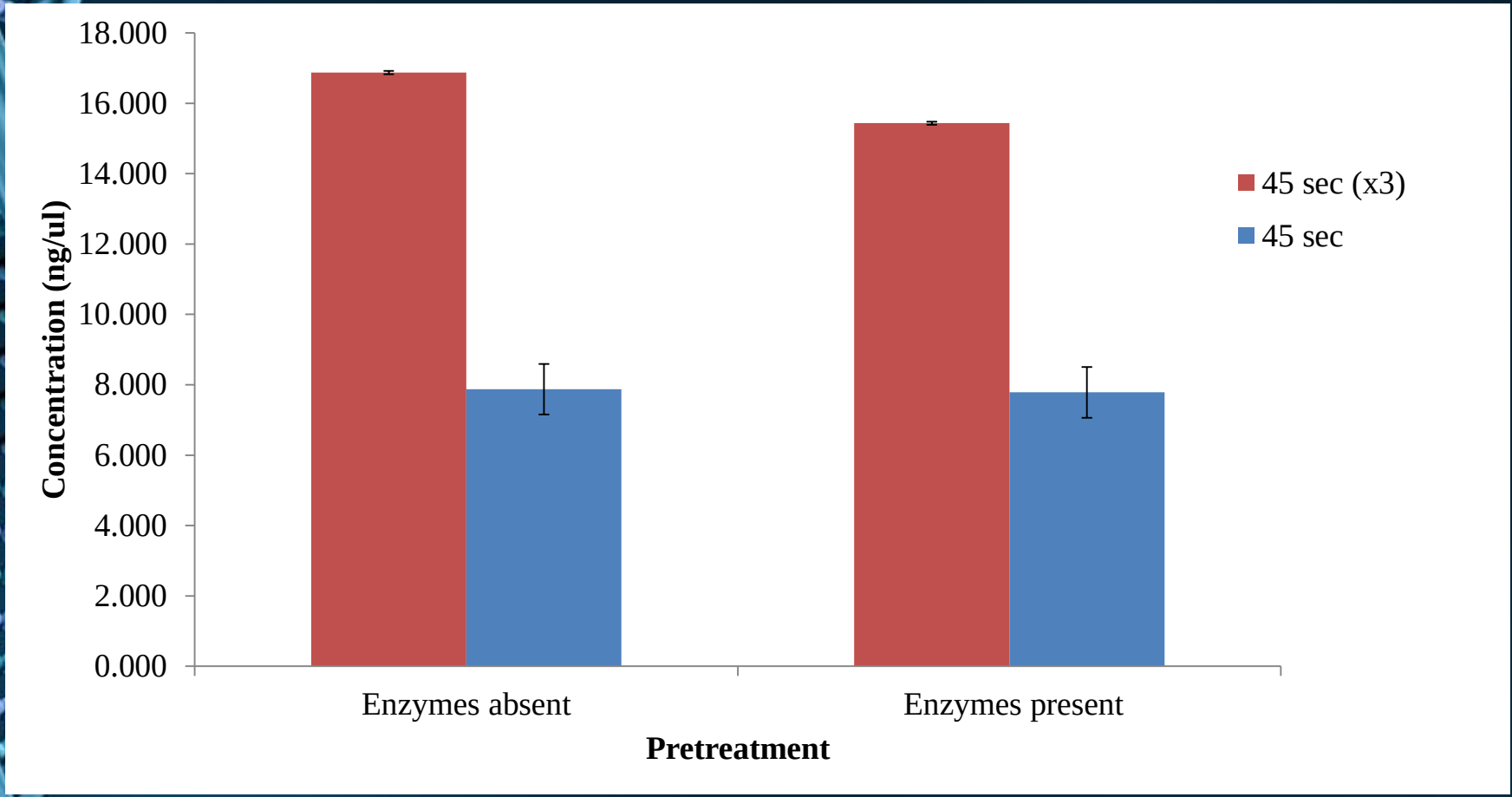


Methods

3. Isolate genomic DNA from the serial dilutions

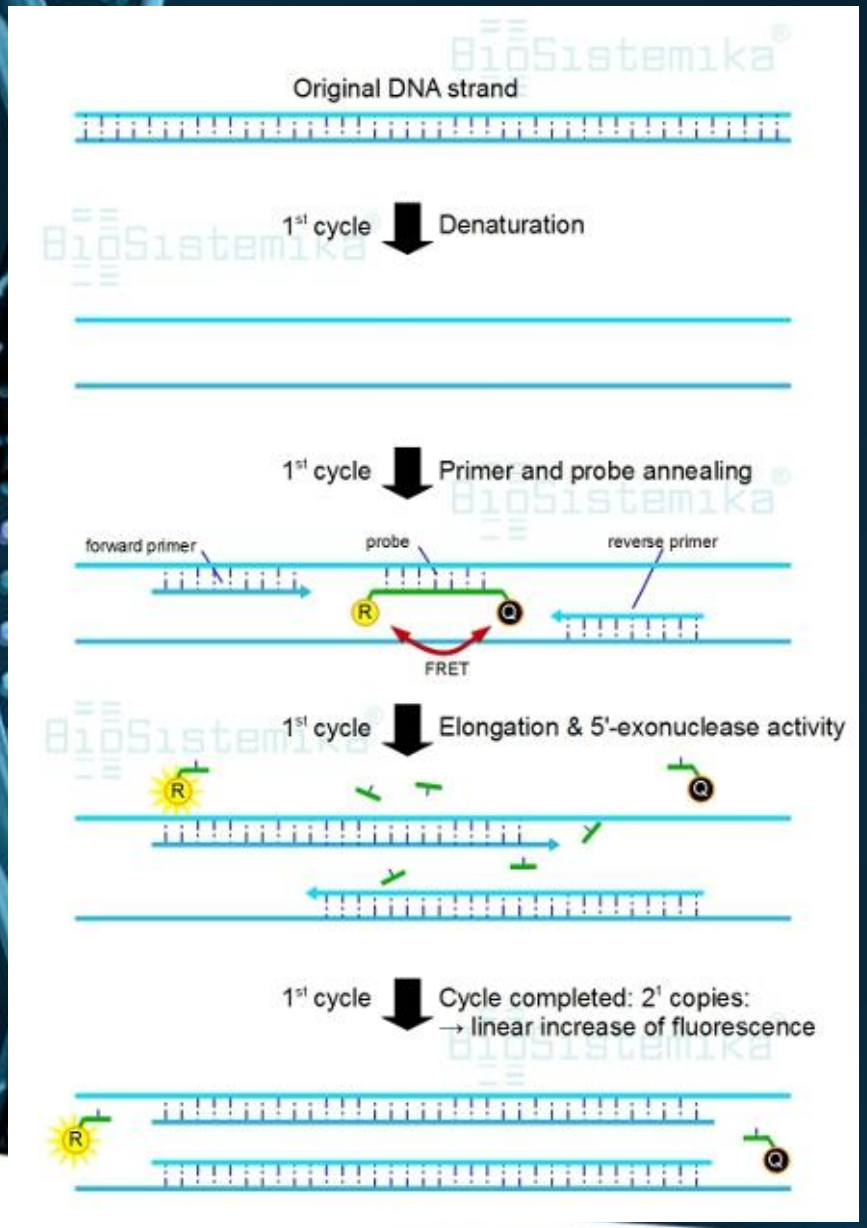
4. Optimization of cell lysis (gram positive)
   - Lysozyme
   - Proteinase K
   - Cell Disruption by bead beating

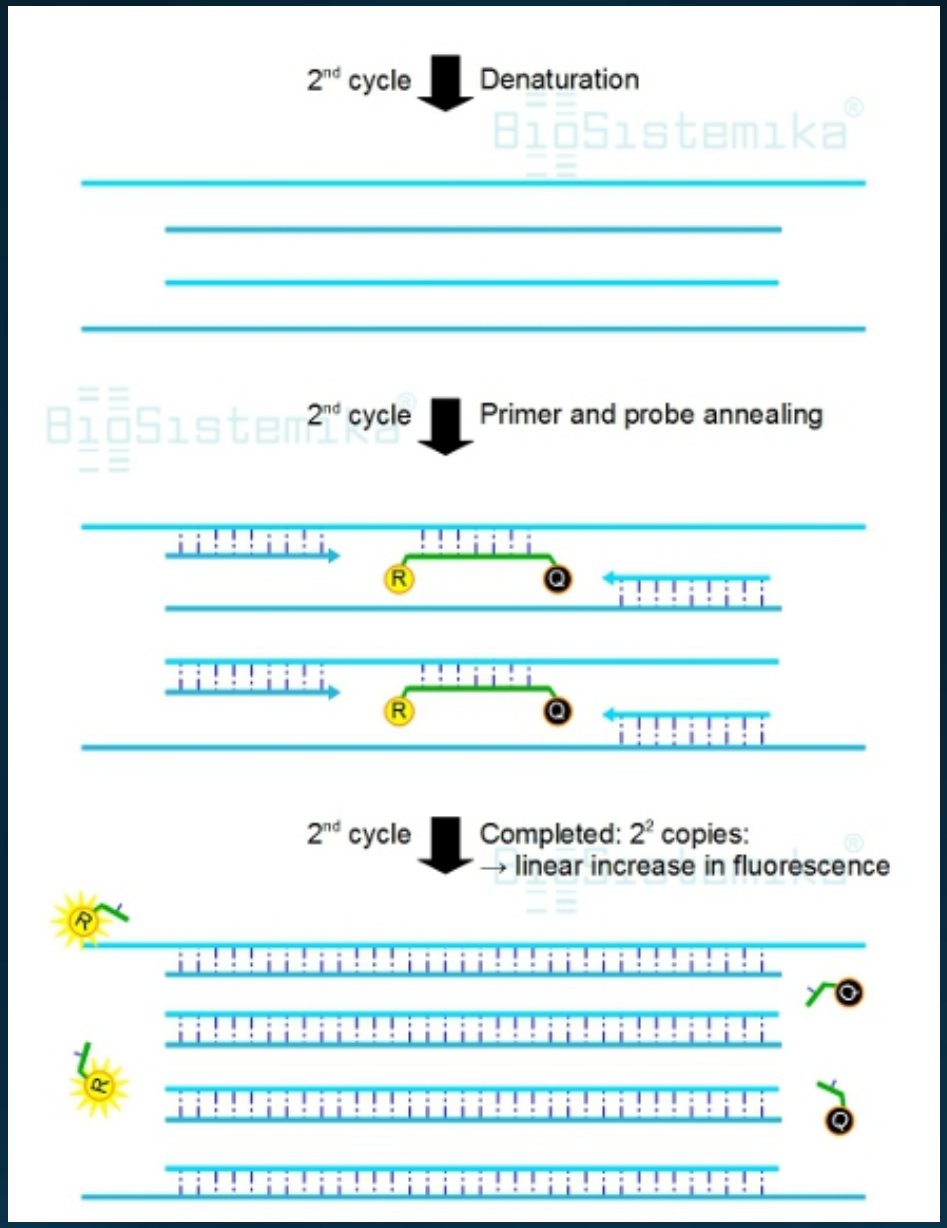
Cell Lysis Results



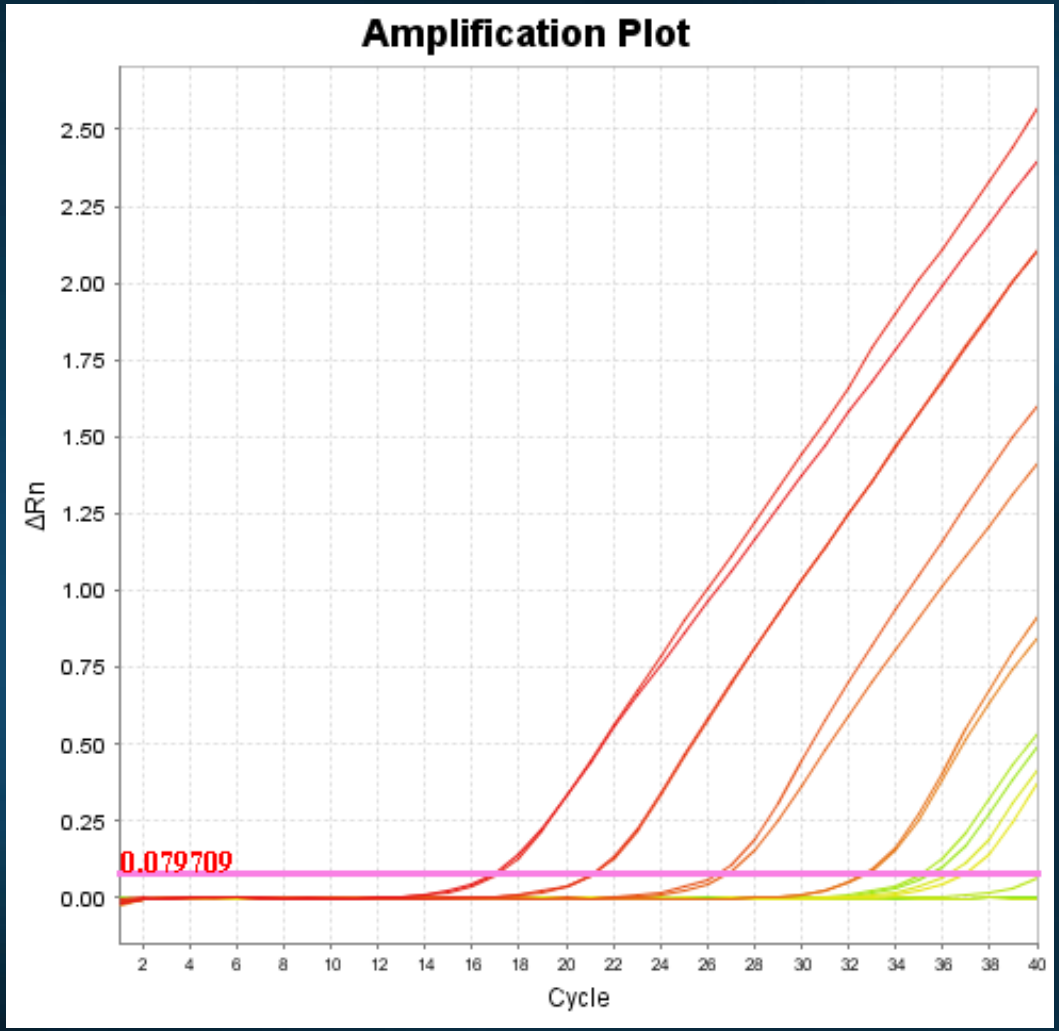
Comparing pretreatments for gram-positive bacteria (*Pediococcus acidilactici*): Lysozyme and Proteinase K and cell disruption by bead beating. There was a significant difference between cell disruption treatments ($p = 0.05407$), but no significant difference between the pretreatments with and without enzymes ($p = 0.8537$).

5. Conduct qPCR (TaqMan) on the serial dilutions

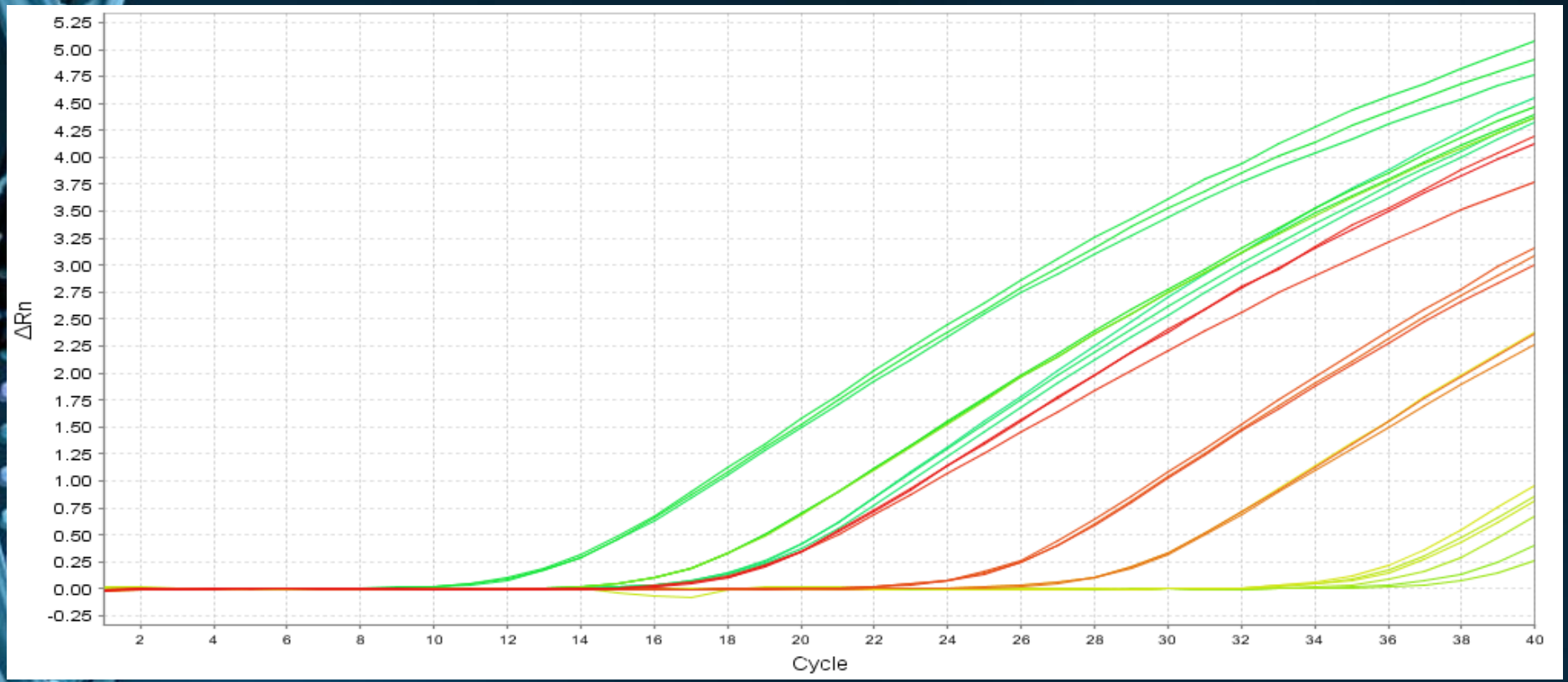




6. Create a standard curve to enumerate the cells in an unknown sample

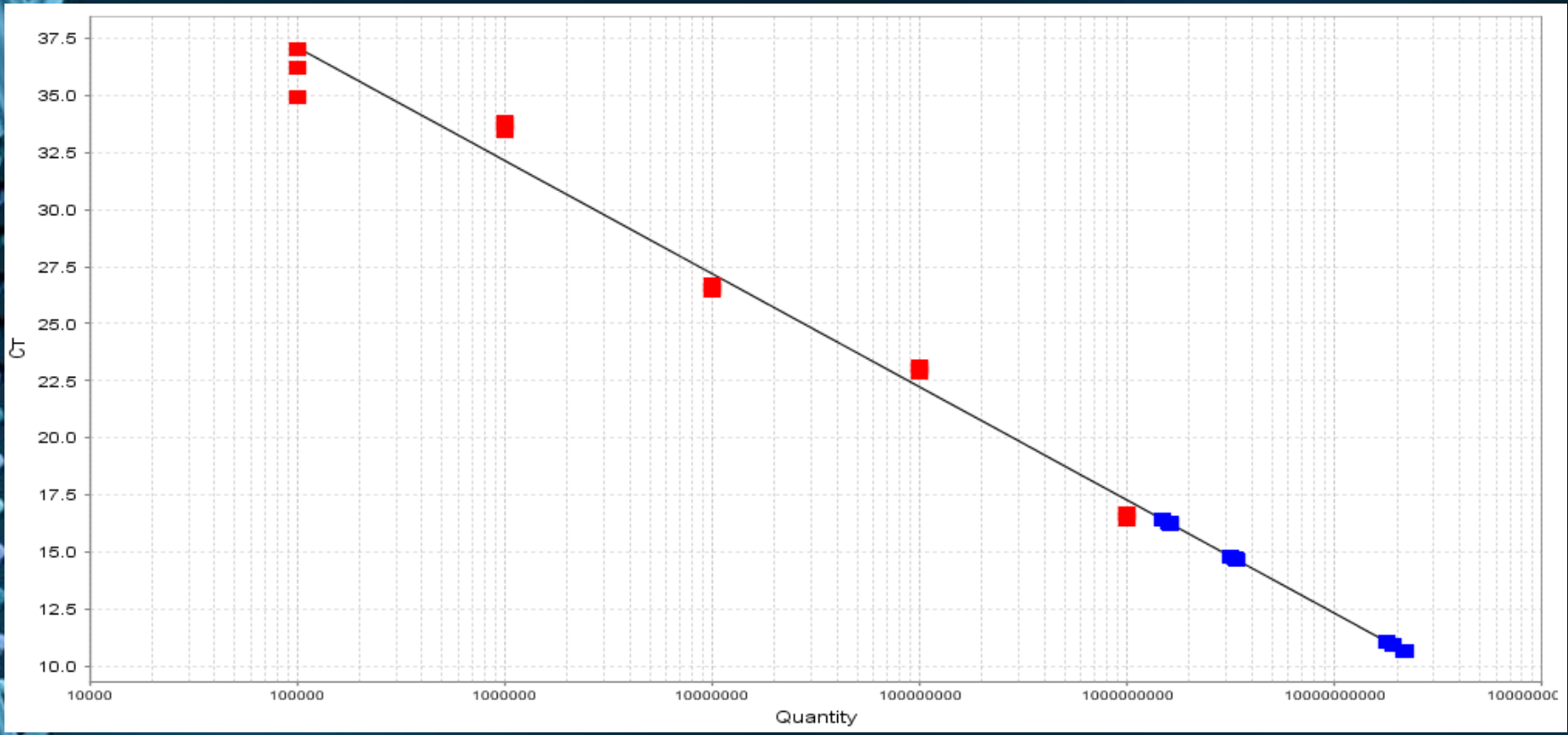


Results: qPCR Amplification Curves



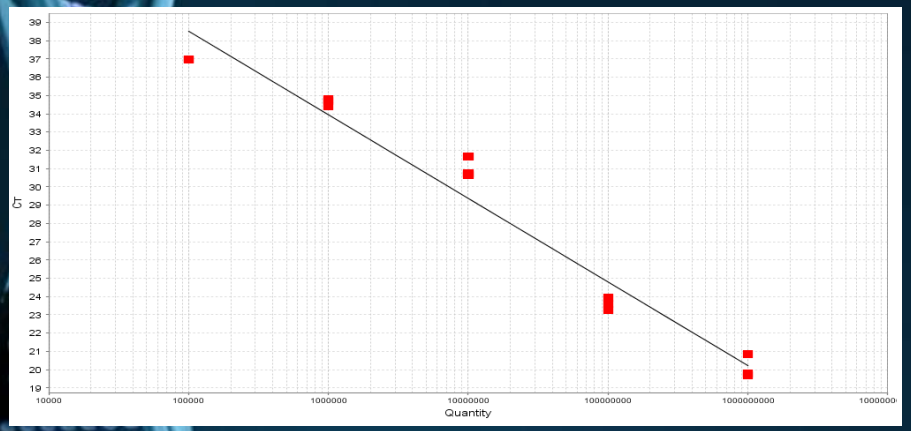
Amplification plot for *Lactobacillus plantarum* and lyophilized cell samples of unknown composition. Results show there is a high level of amplification in the lyophilized samples.

Results: qPCR Standard Curve with unknowns

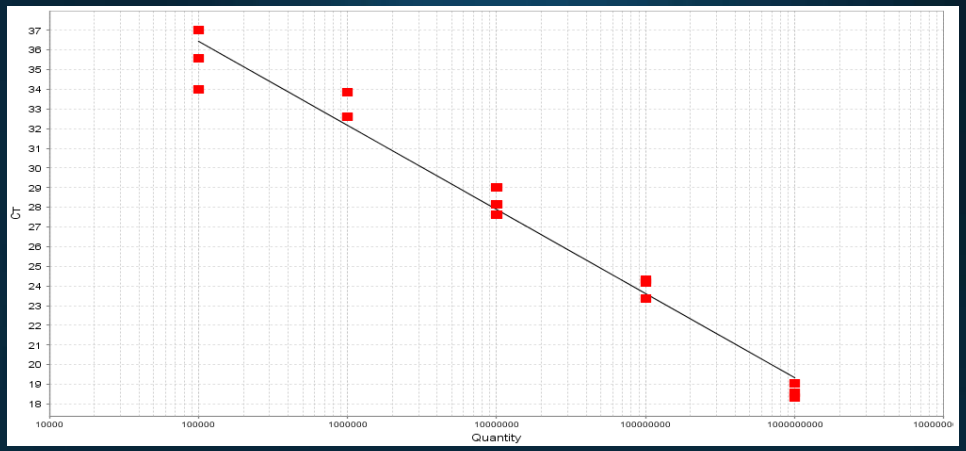


Standard curve for *Lactobacillus plantarum* and lyophilized cell samples of unknown composition. Results show there is a high level of *Lactobacillus plantarum* bacteria in the lyophilized samples.

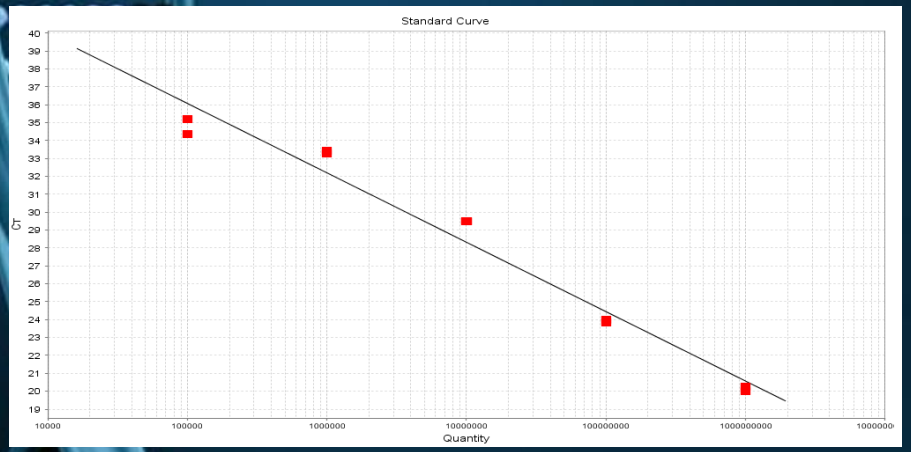
Results: All qPCR Standard Curves



Bacillus amyloliquifaciens



Lactobacillus plantarum



Pediococcus pentosaceus

Conclusion

- Long bead beating times result in more efficient DNA isolation
- Standard curve protocol for probiotic bacteria was established and optimized
- The results for our unknowns were outside our standard curve, so we should dilute samples and repeat quantification

Acknowledgements

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- Rodríguez-Lázaro, D. and Hernández, M. (2013). Real-time pcr in food science current technology and applications. *Horizon Scientific Press* 25-28
- Hoffman, L. and Jarvis, B. W. (2003). DNA purification from hard-to-lyse bacteria using Ready-Lyse™ Lysozyme and the MasterPure™ DNA purification kit. *Epicentre* 3

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Introduction and Background

Probiotics are live microorganisms, like bacteria and yeast, which are thought to provide health benefits when ingested. Large manufacturers produce blends of probiotics for supplementing the diets of agriculturally important animals. Commercial probiotic products are labeled with the number of cells present at the time of manufacture. Our assignment was to develop an assay which can be used to quantify the number of specific lactic acid bacteria present in certain probiotic products, thus verifying the number reported on the product labels. This quantification process involves isolating genomic DNA (gDNA) from the given samples and then running the DNA through a quantitative polymerase chain reaction (qPCR). We optimized gDNA isolation by 1) treating these gram-positive bacteria with lysozyme and proteinase K, and 2) increasing the length of cell disruption by bead beating (FastPrep). Results showed that gDNA yields were improved by longer FastPrep treatments, but not by enzyme treatments. Quantitative polymerase chain reactions (qPCR) on isolated gDNA allow researchers to make copies of a short target DNA sequence and monitor its amplification in real time through the use of a fluorescent probe. Using this method, we created standard curves for four species of bacteria which allowed us to correlate cell quantity to threshold cycle. These standard curves allow us to quantify the number of each bacteria present in probiotic mixes of unknown composition.

Objective: Develop an assay which can be used to quantify the number of specific lactic acid bacteria present in probiotic products.

Probiotic Species: *Pediococcus acidilactici, Pediococcus pentosaceus, Lactobacillus plantarum, Bacillus methylotrophicus*, *Bacillus amyloliquefaciens*

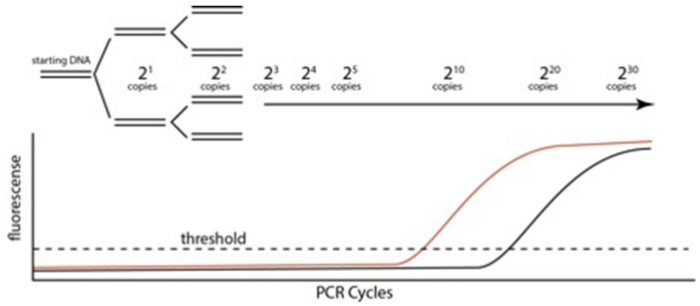
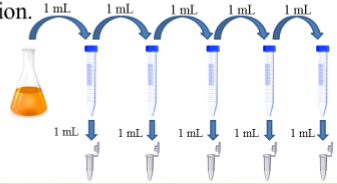


Figure 1. The TaqMan probe is an oligonucleotide double labeled with a reporter fluorophore at the 5' end (reporter dye) and a quencher at the 3' end (quencher dye). In close proximity, the quencher absorbs reporter dye. During the extension phase of the polymerase chain reaction, exonuclease activity cuts DNA and the reporter is detached from the quencher. This separation allows for fluorescence to be expressed by the reporter. As more DNA is amplified, more fluorescence is detected by qPCR.

Methods

1. Grow a 25 mL overnight culture of bacteria in MRS at 35 °C.

2. Spike 10% dextrose with cells and conduct serial dilution.



3. Isolate genomic DNA from the serial dilutions

4. Optimization of gDNA isolation
   - Lysozyme
   - Proteinase K
   - Cell Disruption
5. Conduct qPCR on the serial dilutions to create a standard curve
6. Use the standard curve to enumerate the cells in an unknown mixture

Results

Comparing cell disruption pretreatments of lysing enzymes and bead beating for gram-positive bacteria *Pediococcus pentosaceus*

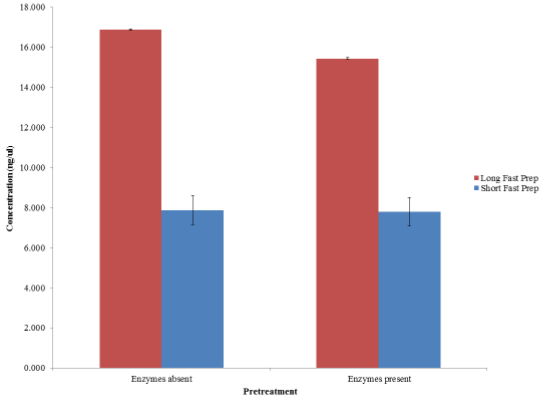


Figure 2. Comparing pretreatments for gram-positive bacteria (*Pediococcus pentosaceus*): Lysozyme and Proteinase K and cell disruption by bead beating. The "Long FastPrep" time indicates the bacteria experienced 135 seconds of cell disruption and the "Short FastPrep" indicates the bacteria experienced 45 seconds of cell disruption by bead beating. There was a significant difference between cell disruption treatments ($p = 0.05407$), but no significant difference between the pretreatments with and without enzymes ($p = 0.8537$).

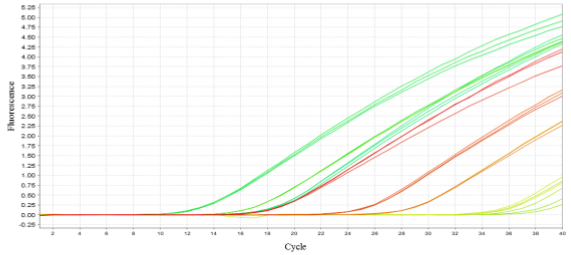


Figure 7. Amplification plot for *Lactobacillus plantarum* and lyophilized cell samples of unknown composition. Results show there is a high level of amplification in the lyophilized samples.

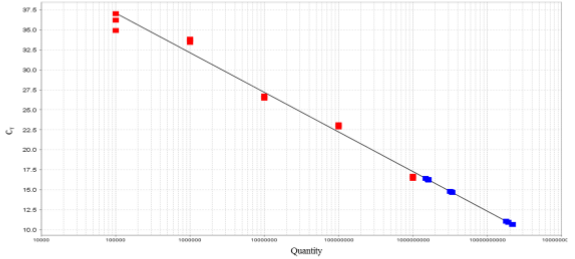


Figure 8. Standard curve for *Lactobacillus plantarum* and lyophilized cell samples of unknown composition. Results show there is a high level of *Lactobacillus plantarum* bacteria in the lyophilized samples.

Sources

1. Rodríguez-Lázaro, D. and Hernández, M. (2013). Real-time pcr in food science current technology and applications. *Horizon Scientific Press* 25-28
2. Hoffman, L. and Jarvis, B. W. (2003). DNA purification from hard-to-lyse bacteria using Ready-Lyse™ Lysozyme and the MasterPure™ DNA purification kit. *Epicentre* 3

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