PROBIOTIC STRAIN LEVEL VIABLE COUNT USING VIABILITY PCR

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Background

Recent years have witnessed a rapid increase in the global probiotic market size which was valued at USD 58.17 billion in 2021 and is expected to reach USD 111.21 billion in 2030 (Grand-View-Research-Inc, 2022). Probiotic health benefits are strain specific and are dose dependent (Klein et al., 2010; Sánchez et al., 2017; McFarland et al., 2018). Thus, it is vital to verify that a probiotic product meets label claims of strain content and viable cell counts to achieve efficacy. Furthermore, probiotic products should maintain the recommended dose of viable cells throughout their shelf life (Tripathi and Giri, 2014; Kolaček et al., 2017; Sánchez et al., 2017). Proper methods are required to determine the viable counts throughout the shelf life of probiotic products.

Plate count methods are currently the standard methods used for viable count determination (Hill and Slack, 1904; Hill, 1908; Breed and Dotterrer, 1916; Davis, 2014). Plate count methods measure the ability of cells to proliferate to form colonies on growth media, with the results expressed as Colony Forming Units (CFU) (USP, 2018). These methods are known to be time consuming, laborious, non-specific, variable, and require special growth media and growth conditions (Hansen et al., 2018; Jackson et al., 2019). Furthermore, because plate count methods measure cultivability, they are unable to quantify viable but non culturable (VBNC) cells (Fiore et al., 2020). VBNC cells have intact cell membranes, maintain some metabolic activity, and can reproduce and interact with the host under favorable conditions of the gut (Fiore et al., 2020). CFU count is thus considered an estimate, not a true cell count (USP, 2018).

Another method for probiotic viable count determination is viability real-time PCR (v-PCR) (García-Cayuela et al., 2009; Kramer et al., 2009; Shehata and Newmaster, 2021). In v-PCR, cells are pre-treated with a viability dye such as propidium monoazide (PMA) or its modified form, PMAxx. This viability dye has the ability to cross cell membranes of dead or membrane damaged cells only, then irreversibly intercalates to DNA, rendering DNA from dead or membrane damaged cells unreactive in PCR (Fittipaldi et al., 2012). v-PCR methods rely on membrane integrity and therefore, can quantify VBNC cells, which cannot be enumerated by plate count methods (Davis, 2014). v-PCR methods are sensitive, accurate, fast and simple. Additionally, they are targeted methods thus they enable specific enumeration at the species or strain level. This property is extremely useful when enumerating individual strains in multi-strain blends. Here, we present the development and validation of a v-PCR method for the strain specific enumeration of one of the most commonly used probiotic strains in probiotic products, strain Lacticaseibacillus rhamnosus GG (Shehata and Newmaster, 2020; 2021).

Materials and Methods

To design a strain-specific qPCR assay, a unique sequence region was identified in the genome of *L. rhamnosus* GG compared to closely related strains. The assay was first validated following the guidelines for validation of qualitative real-time PCR methods for molecular diagnostic identification of probiotics (Broeders et al., 2014; Shehata et al., 2019; Shehata and Newmaster, 2020). The specificity of the assay was confirmed using 22 target samples and 28 non-target strains including other *L. rhamnosus* strains such as HN001, Lr-32, UALr-06, HA-111, HA-114 and R0011 to ensure strain level specificity. This viability real-time PCR method was then validated for viable count determination of *L. rhamnosus* GG, following the guidelines for quantitative real-time PCR (Bustin et al., 2009; Broeders et al., 2014; Shehata and Newmaster, 2021). The optimal viability dye treatment was determined by evaluating the ability of different concentrations of the viability dye to inactivate DNA from heat killed cells, then multiple bead-beating protocols were evaluated to find the most effective protocol to liberate DNA. The efficiency and linear dynamic range of the methods were assessed. Finally, the assay performance was compared to a plate count method.

Results and Discussion

Bioinformatic analysis identified a unique sequence region in the genome of *L. rhamnosus* GG. A strain specific real-time PCR assay was designed to target the identified region which was found to code for a hypothetical protein (Shehata and Newmaster, 2020). Evaluating the specificity of *L. rhamnosus* GG assay showed that all 22 target samples successfully amplified with a mean Cq of 23.66. Non-target samples including six *L. rhamnosus* strains (HN001, Lr-32, UALr-06, HA-111, HA-114 and R0011) either did not show any amplification or showed late inconsistent amplification after 38 cycles. Thus, a threshold Cq value was set to 32 cycles (Figure 1).

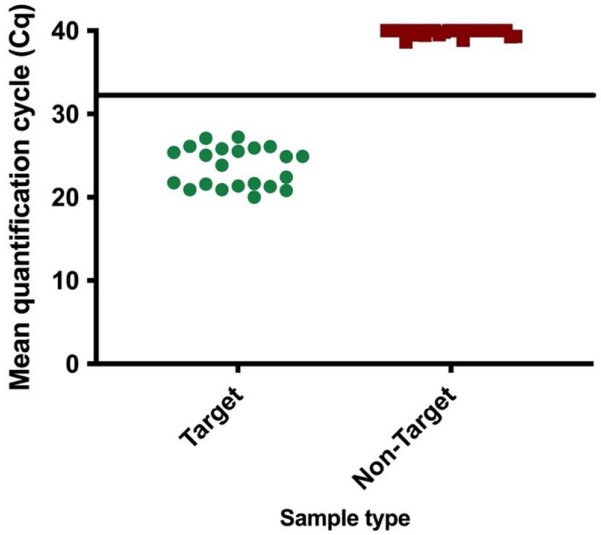

Figure 1: Evaluating the analytical specificity of *Lactobacillus rhamnosus* GG strain specific identification method. Specificity was evaluated using 22 target and 28 non-target samples. A Cq value of 40 was given to samples that did not show any amplification for inclusion in the graph. The vertical line at a Cq value of 32 cycles denotes the threshold Cq (Shehata and Newmaster, 2020).

Multiple viability dye (PMAxx) treatments and DNA liberation protocols were evaluated. PMAxx at a final concentration of 50 M was selected as an efficient concentration for inactivating DNA from dead cells (Figure 2). Bead beating for 5 min at 3000 rpm was selected as an efficient method for DNA liberation.

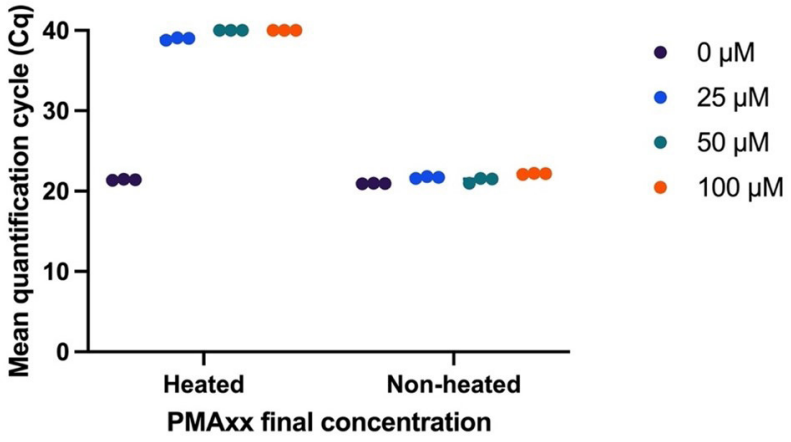

Figure 2: Optimization of viability dye treatment for *L. rhamnosus* GG enumeration method. No amplification was observed from heat-killed cells treated with 50 M and 100 M of PMAxx (represented by a Cq value of 40 for inclusion in the graph) (Shehata and Newmaster, 2021).

When evaluating reaction efficiency, the linear dynamic range was established between 3.94* 106 to 3.94* 103 genomes and reaction efficiency was 102%, 98%, and 93% in three independent trials, with R square values of 0.9956, 0.9966 and 0.9988 (p value < 0.0001) (Figure 3). Ideal reaction efficiency for a quantitative real-time PCR is between 90% and 110% with R2 values ≥ 0.98 (Broeders et al., 2014). Thus, *L. rhamnosus* GG enumeration method described here met the criteria for ideal reaction efficiency.

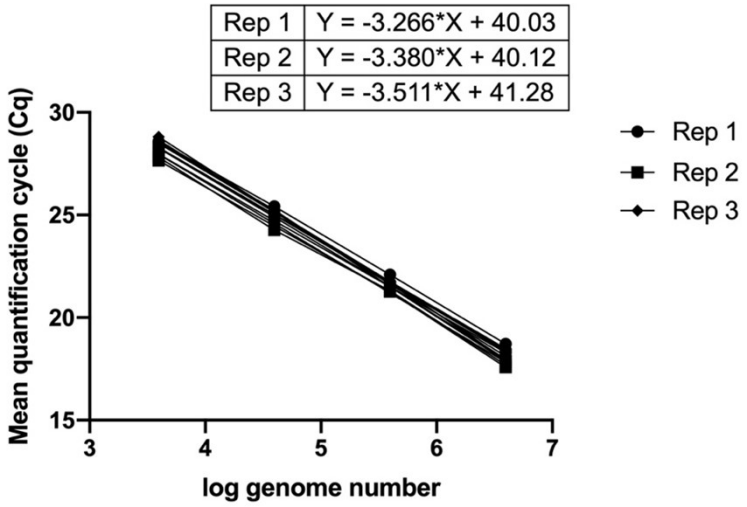

Figure 3: Evaluating reaction efficiency for *L. rhamnosus* GG enumeration method (Shehata and Newmaster, 2021).

The performance of the method for *L. rhamnosus* GG enumeration was compared to the gold standard method, plate count method, using 10 mono-strain samples. A high correlation was observed between the counts determined using the two methods (Pearson r = 0.8451, P-value = 0.0021). The applicability of the method for multi-strain products containing non-active ingredients was evaluated using 13 experimental mixtures of strain *L. rhamnosus* GG. The method gave comparable cell count estimates compared to plate count methods. The average relative difference between both methods for all 23 samples was 18.5% (Figure 4).

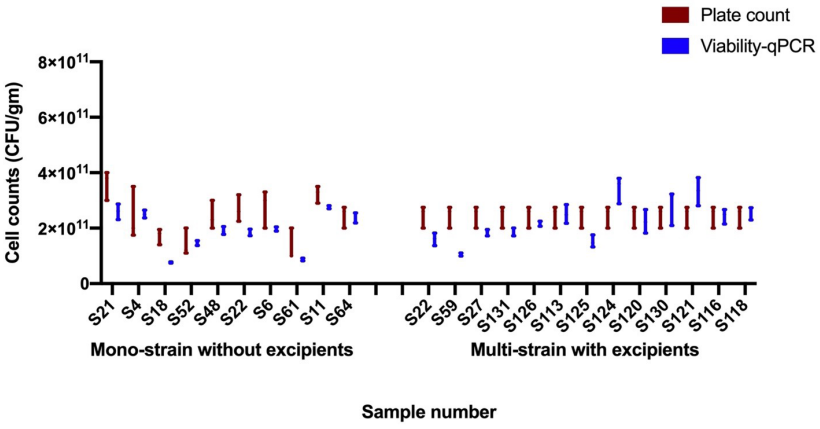

Figure 4: Comparing the performance of *L. rhamnosus* GG v-PCR enumeration method to plate count method using ten mono-strain samples and 13 multi-strain samples with non-active ingredients. Shown are box plots from three replicate values (Shehata and Newmaster, 2021).

Conclusions

The method described here for the strain specific enumeration of *L. rhamnosus* GG demonstrated high specificity, with reaction efficiency in the ideal range. The method is able to achieve both identification and viable count determination of strain *L. rhamnosus* GG, and offers a much shorter time to results compared to plate count methods. Furthermore, the method is applicable to mono-strain as well as multi-strain samples and to samples mixed with other non-active ingredients. This method will enable fast and precise viable count determination of strain *L. rhamnosus* GG as a raw ingredient as well as in finished products and can thus support quality control measures to achieve compliance in probiotic products.

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