Comparison of Quantitative Real-time PCR (qPCR) and Droplet Digital PCR (ddPCR) for Quantification of *Lactobacillus reuteri* in Human Feces

by

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Abstract

The human gut microbiota, a complicated microbial community consisting of trillions of microorganisms, has been recognized as of great importance for maintaining human health. To establish direct linkages between a certain group of gut microorganisms and the physiological status of the host, it is critical to quantify their abundance with high sensitivity, accuracy, and reproducibility. Quantitative real-time PCR (qPCR) has been widely used in the absolute quantification of microorganisms by comparing PCR cycle numbers with those of a standard curve. Recently, the development of droplet digital PCR (ddPCR) has demonstrated the potential to handle samples with a complex background without standard curves. The goal of this work was to compare absolute quantification of a specific bacterial strain (*Lactobacillus reuteri* DSM 17938) in human stool between qPCR and ddPCR with three commonly used DNA extraction methods (QIAamp Fast Stool DNA Kit [QK], phenol chloroform [PC], protocol Q [PQ]). DNA extracted using QK and PQ had acceptable quantity and high quality while PC produced DNA with highest concentration but lower purity. Compared to the other two methods, PQ recovered the most substantial proportion of *L. reuteri* cells from feces. Generally, reproducibility was better in ddPCR than qPCR with methods QK and PC, but comparable with PQ. Within the detectable range, both qPCR and ddPCR presented better linearity with DNA extracted using methods QK and PQ than PC. For qPCR, the limit of detection (LOD) was 3.95 Log_{10}, 4.86 Log_{10}, 4.11 Log_{10} CFU/g feces with QK, PC and PQ. ddPCR exhibited a lower limit of quantification (LOQ) when compared to qPCR. The LOQ was 4.30 Log_{10} CFU/g feces when QK and ddPCR were combined and was slightly higher in use of PQ and qPCR (4.50 Log_{10} CFU/g feces). However, the cost of ddPCR per unit was 3 times higher than qPCR and is more time-consuming (6.5 h vs. 2.5 h). Therefore, the combination of PQ and qPCR is suggested as the best to detect *L. reuteri* in fecal samples.

The second goal of my project was to use the information above and design strain/lineage-specific qPCR systems for the absolute quantification of *L. reuteri* PB-W1 and DSM 20016^{T} in fecal samples. To achieve this, strain/lineage-specific primers were designed using a database approach. The LOD for PB-W1 measurement was 3.05 Log_{10} CFU/g feces, which was the lowest level reached in the field, and for DSM 20016^{T}, it was 2.96 Log_{10} CFU/g feces, which was equally decent. Overall, this work successfully established approaches for a quantitative, selective, accurate, and sensitive quantification of bacterial strains in human fecal samples based on qPCR and ddPCR and provided information to select the appropriate methods for measurement and DNA extraction.

Preface

This thesis is an original work by Junjie Li. The research project, of which this thesis is a part, received research ethics approvals from The Health Research Ethics Board - Biomedical panel of the university of Alberta (Pro00077565).

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List of abbreviations

qPCR: quantitative real-time PCR

ddPCR: droplet digital PCR

FISH: fluorescence in situ hybridization

rRNA: ribosomal RNA

NGS: next-generation sequencing

WHS: Whole Metagenomic Sequencing

LOD: limit of detection

LOQ: limit of quantification

CV: coefficient of variation

SD: standard deviation

Tm: melting temperature

Cq: quantification cycle

1. Introduction

1.1 The gut microbiota

Humans live with a microbiota in a wide range of bodily niches, of which the gut microbiota harbors the highest numbers of microorganisms (Backhed *et al.*, 2005), reaching approximately 10^{14} total microbial cells per human individual (Krumbeck *et al.*, 2018). The human gut microbiota is a highly complex microbial community consisting of bacteria, archaea, fungi, protozoa, and viruses (Forster *et al.*, 2019; Yatsunenko *et al.*, 2012). The dominant microbes in this ecosystem are bacteria, whose total number associated with the human body is close to the number of human cells (Sender *et al.*, 2016). *Firmicutes* and *Bacteroidetes* are considered to be the most abundant phyla, and over 1000 bacterial species residing in the colon have been identified (Rajilic-Stojanovic and de Vos, 2014). Forming a mutualistic relationship, the microbiota is provided with an anaerobic environment by the gastrointestinal (GI) tract of the host, while fermenting indigestible polysaccharides and producing nutrients and energy in return (Backhed *et al.*, 2005). However, the gut microbiota has also been implicated in many human diseases (Fujimura *et al.*, 2016; Kostic *et al.*, 2013; Nishida *et al.*, 2018; Wang *et al.*, 2011). Therefore, exploring the composition and functions of the gut microbiota is crucial for understanding the host biology and health.

1.2 The role of gut microbiota in host biology

1.2.1 Roles of the gut microbiota in health status

The functional characteristics of the gut microbiota have been studied for decades. Even though we are unsure of the whole picture, there is evidence revealing that the gut microbiota influences the host health in diverse aspects, including shaping the immune system (Geva-Zatorsky

et al., 2017), colonization resistance against pathogens (Buffie *et al.*, 2015), nutrition utilization (Conlon and Bird, 2014), and neural development (Rogers *et al.*, 2016). These specific functionalities of the gut microbiota have been vastly demonstrated by comparing germ-free with conventional mice (Brown *et al.*, 2017; M *et al.*, 2014; McVey Neufeld *et al.*, 2015). In humans, roles of the gut microbiota have been confirmed in studies related to fecal microbiota transplantation (DeFilipp *et al.*, 2018). This treatment is emerging owing to its strong effectiveness in curing recurrent *Clostridium difficile* infection (van Nood *et al.*, 2013). For other diseases such as obesity, active ulcerative colitis, and autism, its therapeutic potential has been shown in clinical studies (Kang *et al.*, 2017; Moayyedi *et al.*, 2015; Paramsothy *et al.*, 2017; Ridaura *et al.*, 2013), but not well established yet. Conversely, the host, during evolution, has gained the ability to interact with the gut microbiota by microbial sensing, immune response, etc. (Thaiss *et al.*, 2016). This sustained partnership between the host and the gut microbiota considerably contributes to the maintenance of homeostasis.

1.2.2 Roles of gut microbiota in disease

The change in gut microbiota has been implicated in not only intestinal diseases such as colorectal cancer (Kostic *et al.*, 2013) and inflammatory bowel disease (Nishida *et al.*, 2018) but also extraintestinal diseases, including but not limited to cardiovascular disease (Wang *et al.*, 2011), and diabetes (Karlsson *et al.*, 2013). While there is still significant uncertainty on whether the change of gut microbiota leads to diseased state or it is the opposite direction, studies have been conducted continuously to reveal the contribution of gut microbiota to some particular diseases. For example, scientists have reported the association between the gut microbiome and sporadic colon cancer. By comparing the colonic microbes and metabolites of African Americans and native

Africans, a lower risk of colon cancer in the latter group was connected with more health-promoting metabolites such as butyrate and less carcinogenic products such as secondary bile acids produced by gut bacteria (Ou *et al.*, 2013). In addition, neonatal microbiota dysbiosis has been associated with the development of asthma later in life. Lower relative abundance of bacterial taxa such as *Bifidobacterium*, and *Akkermansia* and higher relative abundance of certain fungi such as *Candida* in the gut microbiota of infants resulted in a high risk of childhood atopy and asthma, potentially through influencing $CD4^+$ cell population and function (Fujimura *et al.*, 2016).

With a wide use of animal models, especially gnotobiotic or humanized mouse, scientists have been allowed to study the microbial perturbation under controlled conditions, and thus help to investigate causality of alterations in the gut microbiome related to pathophysiologies (Kim *et al.*, 2017; Sampson *et al.*, 2016; Schaubeck *et al.*, 2016). For instance, scientists transformed fecal microbiota from twin pairs that were discordant for obesity to germ-free mice and observed substantially greater increases in body mass in the group receiving obese twin's microbiota than the mice harboring lean twin's microbiota. This finding was linked to an increased production of short-chain fatty acid in the lean group and an increased metabolism of branched-chain amino acids in obese group (Ridaura *et al.*, 2013). In a recent study, researchers established the relationship between a multiple sclerosis-associated microbial profile (e.g., the abundance of the genus *Sutterella*) and multiple sclerosis-like autoimmune disease using a mutant mouse model that was humanized by fecal transplantation (Berer *et al.*, 2017). The findings demonstrated the possibility of characterizing the specific roles of gut microbiota in neuro-inflammatory diseases (Berer *et al.*, 2017).

1.2.3 Factors affecting the gut microbiota

Evidence has shown that the complex microbial community in the digestive tract can be affected and shaped functionally and compositionally, mainly through environmental factors (e.g., diet) and host genetics (Benson *et al.*, 2010). However, the genetic factor seems to be less important according to outcomes shown in studies (Carmody *et al.*, 2015; Rothschild *et al.*, 2018). Additionally, medicine is a key modulator of the gut microbiome with antibiotics being mostly studied. Antibiotics have been widely used for infection treatment for decades, and one of the influences on the gut is decreasing the diversity of microbiota (Dethlefsen and Relman, 2011). Even though antibiotics are implicated in the initiation of some diseases such as *Clostridium difficile* infection (Seekatz *et al.*, 2014), whether it is harmful to gut health is still unclear as human responses to antibiotics varied among studies (Reijnders *et al.*, 2016). Other factors such as age and lifestyle also have impacts on the intestinal ecosystem while only to a limited degree (Falony *et al.*, 2016).

1.2.4 Dietary effect on the gut microbiota

Dietary shift has is capable of changing the gut microbiota within a short term (O'Keefe *et al.*, 2015). Thousands of years ago, the way humans obtain nutrition was changed by the invention of farming and livestock breeding, and more recently, by the industrialized food production (Fuller *et al.*, 2014). This shift reduced gut microbiota diversity within the past decades (Deehan and Walter, 2016). The key driver was believed to be the declined intake of carbohydrates available to gut microbes (Deehan and Walter, 2016). These edible but non-digestible carbohydrate polymers are often referred to as dietary fiber, which includes resistant starch, inulin, arabinoxylan, and pectin (Makki *et al.*, 2018). They can be fermented by certain types of intestinal microorganisms

and release specific products such as short-chain fatty acids (Wong *et al.*, 2006), trimethylamine, and indole propionic acid (de Mello *et al.*, 2017), which deliver certain health-promoting effects to the host (Byndloss *et al.*, 2017; de Mello *et al.*, 2017; Frost *et al.*, 2014). High-protein and high-fat contents, which are main characteristics of the modern diet, have also been connected to the increased release of detrimental metabolites in the gut, and thus deliver microbiota-associated diseases. For example, a previous study has demonstrated that the ingestion of L-carnitine, abundant in red meat, increased the production of trimethylamine-N-oxide and promoted atherosclerosis with enriched proportion of *Prevotella* in the gut microbiota (Koeth *et al.*, 2013). Besides, a dietary intervention study uncovered the correlation between a high-fat diet and an increased risk of colon cancer characterized by shifted microbial metabolites in the gut, such as boosted secondary bile acids and suppressed butyrate (O'Keefe *et al.*, 2015).

1.2.5 Other strategies to modulate the gut microbiota

Some living microorganisms are also considered to confer beneficial effects when administrated in adequate amounts, known as probiotics (Hill *et al.*, 2014). Currently commercially available probiotic products mainly contain bacteria of the genera *Bifidobacterium* and *Lactobacillus*, and some products have been shown to exert therapeutic effects in specific applications (Ahl *et al.*, 2016; Greifova *et al.*, 2017; Heuvelin *et al.*, 2009). However, the colonization of most bacterial strains has been shown to be temporary, which brings out the concern about whether they can effectively deliver beneficial effects without persistence in the gut (Charbonneau *et al.*, 2013; Frese *et al.*, 2012; Rattanaprasert *et al.*, 2014). Moreover, the introduction of a probiotic strain did not often lead to notable changes of the gut microbiota

(Laursen *et al.*, 2017; Maldonado-Gomez *et al.*, 2016), potentially indicating that compositional alterations is not a major mechanism by which these live microbes benefit host health.

Other options such as prebiotics and antibiotics can also be considered to regulate the intestinal microbiota. Consuming prebiotics stimulates certain groups of gut microorganisms and promotes health likely by boosting short-chain fatty acid production and lowering luminal pH (Holscher, 2017). Antibiotics is still one of the most effective tools for controlling infections and removing pathogenic microbes. However, one should be careful when using them because the indigenous microbiota could also be affected unintendedly and significantly (Jernberg *et al.*, 2010). Additionally, a more dramatic strategy, which is known as fecal microbiota transplant, replaces the whole gut microbiota of a patient with a new one from healthy donors. This method is one therapeutic option for refractory *Clostridium difficile* infection but not widely practiced due to limited knowledge on mechanisms and unknown risks (Kelly *et al.*, 2012).

1.3 Characterization of the gut microbiota

Since the 19th century when pioneers first touched this field, study of the human microbiota has experienced a vast development, which has been driven by evolutionary advancement of technology and methodology, including the developments of anaerobic microbiology and molecular biology in the 20th century, and breakthroughs in genomics and DNA sequencing in the past two decades (McPherson, 2014). There is a wide range of approaches that have been applied to study the gut microbiota: culture, microarrays, community fingerprinting methods such as denaturing gradient gel electrophoresis (T/DGGE) and terminal restriction fragment length polymorphism (T-RFLP), etc. (Walker, 2016).

1.3.1 Sequencing-based techniques

DNA sequencing techniques have completely revolutionized the study of the gut microbiota. This is a highly innovative field that constantly produces novel technologies with Sanger automated sequencing being a successful pioneer (Escobar-Zepeda *et al.*, 2015). Since the twenty first century, next-generation sequencing (NGS) has been emerging as the most powerful and widely used tool for the characterization of gut microbiome without culturing (Escobar-Zepeda *et al.*, 2015). Currently, there are mainly two NGS-based strategies: amplicon sequencing (analyzing PCR amplicons of marker genes, mostly 16S rRNA gene) or Whole Metagenomic Sequencing (WMS) via shotgun metagenomics. Both armed with NGS, the two methods are implemented in different ways. The technique using 16S rRNA gene is based on the PCR amplification of a certain region of the 16S rRNA gene. Primers are often designed to bind to relatively conserved regions of the gene, while the fragments in between are more variable among species. This allows coverage of a large number of taxa, while differences of amplicons enable the identification of specific taxa using the existent rRNA gene databases (Albanese *et al.*, 2015). Shotgun metagenomics, instead of relying on a specific region of genome, enables the analysis of the entire genetic material of the whole microbial community through the sequencing of all the DNA after fragmentation using metagenomic databases.

In the past decade, 16S rRNA gene sequencing developed into a robust and reliable approach to characterize the gut microbiota and dominated the field, substantially expanding the knowledge of the composition of the gut microbiota and its dynamics and response to perturbations (Qin *et al.*, 2010). This technique is cost effective and has a relatively low requirement on the depth of sequencing. However, because of its reliance on PCR amplification, the selection of

primers has a prominent influence, which has been reported to account for the bias about the representation of taxon in some studies (Rintala *et al.*, 2017; Tremblay *et al.*, 2015).

Shotgun metagenomics is a more recent development and advanced method and is capable of producing over 100 times more reads than 16S rRNA gene sequencing. It allows simultaneous compositional taxonomic profiling, genomic characterization with a prediction of the functional potential of microbial communities (Simner *et al.*, 2018). Most profoundly, shotgun metagenomics enables monitoring of changes in the gut microbiota at strain level (Li *et al.*, 2016) while 16S rRNA gene sequencing has a lower resolution with species- or more often genus-level information (Hamady and Knight, 2009). However, WMS requires a much more sophisticated data analysis.

1.3.2 Absolute quantification of the gut microbiota

Even though current sequencing-based methods have revolutionized the field of gut microbiota, there is a major limitation of this kind: they only generate data exhibiting a microbial profile with relative abundance. Without information about total bacteria density per sample, it is hard to have the right biological interpretation of these semi-quantitative values. In a previous study, patients receiving allogeneic stem cell transplantation were observed to experience a relative change from a predominance of commensal bacteria to enterococci. This shift was mainly associated with subsequent development or suffering of gastrointestinal graft-versus-host disease (Holler *et al.*, 2014). However, whether to ascribe the change to increase of enterococci or the reduction of other bacteria remained to be determined. Currently, scientists have recognized this limitation of sequencing techniques, starting to measure sample-specific total cell density to achieve absolute quantification of specific species or strains, for example by applying synthetic

spike-in standards (Stammler *et al.*, 2016) and flow cytometry (Props *et al.*, 2017; Vandeputte *et al.*, 2017).

Another concern about sequencing techniques is the depth bias (Lagier *et al.*, 2018; Lynch and Neufeld, 2015). Current metagenomics analyses are not capable of detecting microbes present at concentrations less than 10^5 cells per gram, which leads to the missing of some critical minority populations in the gut such as pathogen *Salmonella enterica* serovar Typhi (Lagier *et al.*, 2012). Therefore, study of gut microbiota and specific health-associated members requires more sensitive and quantitative methodologies, for example the cultivation-dependent method, Fluorescence in Situ Hybridization, quantitative real-time PCR, and droplet digital PCR, to obtain the absolute quantification of specific microorganisms.

1.3.2.1 Culture-dependent approach

Before the event of molecular methods (e.g. PCR), culture-based methods were primarily used to characterize the gut microbiota, which allowed us to identify new species and look at their functions. The easiest form of microbial cultivation is to use selective media, which can be obtained in many ways, for example the addition of specific nutrients or antibiotics, heat shock, phages, etc. (Lagier *et al.*, 2015). Many selective media for dozens of bacterial groups have been described in the literature and are commercially available such as Rogosa SL agar (Westergren and Krasse, 1978). However, the culture-based technique is highly time-consuming and labor intensive, and most selective media are not perfect due to lack of selectiveness (non-targeted microorganisms can also grow) or over strictness (even targeted microbes cannot grow well). Despite those drawbacks, culture-based techniques have been experiencing a renaissance recently, with scientists arguing that they should be considered more due to the limitations of sequencing

techniques (e.g. as it comes to functional analyses) and the need for cultures for functional studies (Berry *et al.*, 2015; Lagkouvardos *et al.*, 2016; Lawley *et al.*, 2012). Equipped with multiple culture conditions, MALDI-TOF mass spectrometry and 16S rRNA gene sequencing, the culture-dependent technology is reborn as culturomics, which is currently applied as complementarity with metagenomics analysis and will prospectively contribute more in the future (Lagier *et al.*, 2012; Lagier *et al.*, 2016).

1.3.2.2 Fluorescence in Situ Hybridization (FISH)

FISH refers to a molecular method based on probes specifically binding to ribosomal RNA of target cells. Since the probes are fluorescently labeled, when visualized by epifluorescent microscopy, researchers can obtain not only the quantity but also the spatial organization of targets without the need for culturing (Harmsen *et al.*, 2002). Another advantage of this technique is the simultaneous detection of multiple members in samples (Lin *et al.*, 2018; Mark Welch *et al.*, 2017), which is of importance to understand mechanisms by which microbial communities of gut assemble and interactions across members of gut microbiota. However, this technique has low sensitivity as an accurate visual enumeration typically requires around 10^6 bacterial cells per milliliter in each sample (Walker, 2016).

1.3.2.3 Quantitative real-time PCR (qPCR)

qPCR, a well-established method, has been widely employed in the quantification of gut microorganisms (Martinez *et al.*, 2018; Zmora *et al.*, 2018). Compared to culture-based methods, this PCR-amplification-based technique has the advantage of higher specificity and more time saving (De Medici *et al.*, 2003; Sohier *et al.*, 2014). Besides, it has been considered as an effective

and convincing approach to validate the outcomes of 16S rRNA gene sequencing and metagenomic sequencing (Schlaberg *et al.*, 2017; Yu *et al.*, 2017). However, it has been shown by different studies that qPCR still has several limitations: a) it is a relative quantitative method which is potentially affected by PCR efficiency and reliance on external standards (Pinheiro *et al.*, 2012); b) it is susceptible to inhibitors commonly existing in the environmental or fecal samples (Doi *et al.*, 2015; Nechvatal *et al.*, 2008; Yang *et al.*, 2014). Even so, qPCR is generally considered as a reliable method of choice for the identification and detection of members of the gut microbiota.

1.3.2.4 Droplet digital PCR (ddPCR)

For the past few years, ddPCR has drawn attention increasingly as an alternative quantitative method. Unlike qPCR that generates amplicons in a bulk reaction, this technique enables individual amplification of targets in thousands of nanoliter-scale water-in-oil droplets per sample (Hindson *et al.*, 2011). Due to these special features, it was regarded as a more accurate and sensitive quantification of nucleic acid without relying on a standard curve compared to qPCR. Early applications of ddPCR were mainly focused on clinical samples such as blood and tissue. For instance, ddPCR was applied to detect copy number variation of specific genes (Hindson *et al.*, 2011; Miotke *et al.*, 2014), to identify mutations in circulating DNA (Taly *et al.*, 2013), and to measure the frequency of HIV DNA (Strain *et al.*, 2013). Samples from these studies had one feature in common: they all contained rare targets and relatively uniform and simplistic background. With further exploitation, however, ddPCR has been shown the potential to cope with analysis in more complicated samples, for example, the detection and quantification of microorganisms in environmental samples (Cave *et al.*, 2016; Palumbo *et al.*, 2016).

1.4 *Lactobacillus reuteri* as a member of the gut microbiota

L. reuteri is a gram-positive bacterium that colonizes the gut of a wide range of mammals (humans, pigs, rats, etc.) and birds, and the species is clustered in different phylogenetic lineages related to the origin of host (Oh *et al.*, 2010). Some evidence has shown that the specialized adaptations to the intestinal ecosystem of the host indicate the evolutionary process of this species with the host. For example, rodent strains form biofilms on the epithelia of the forestomach in mice through the production of specific surface proteins, and they outcompete non-rodent strains in competition experiments in mice (Frese *et al.*, 2013; Walter *et al.*, 2007). More recently, host adaptation was also experimentally demonstrated for *L. reuteri* isolated from chicken in a competition study (Duar *et al.*, 2017).

1.4.1 Presence in the human gut

L. reuteri is considered as autochthonous to the human digestive tract (Reuter, 2001), and strains isolated from human beings generally cluster together on a phylogeny tree (Oh *et al.*, 2010). However, the ecological characteristics of the species in humans are vaguer than that in other animals, and the prevalence in the GI tract of humans is remarkably low (Walter, 2008), and in human feeding trials, persistence is short and no host adaptation of human strains is detected (Duar *et al.*, 2017). This phenomenon was observed in the industrialized countries, especially when compared to people living in a less modernized area, where *L. reuteri* in the gut can still be detected (Martinez *et al.*, 2015). Nevertheless, host specificity was also observed in *L. reuteri* strains isolated from humans. For example, according to findings from comparative genomic characterization of *L. reuteri*, genes encoding adhesin-like surface protein were deleted largely in human isolates, but those related to utility of glycerol and propanediol were basically maintained,

potentially demonstrating their growth in lumen rather than on epithelial lining (Frese *et al.*, 2011; Walter *et al.*, 2011). This is substantially different from rodent strains which colonize the forestomach of mice through forming biofilm (Frese *et al.*, 2013; Walter *et al.*, 2007).

1.4.2 Probiotic effects of *L. reuteri*

Several beneficial traits of *L. reuteri* have been observed and revealed, including but not limited to production of antimicrobial substances, regulation of the host immune system, and enhancement of the intestinal barrier (Ahl *et al.*, 2016; Greifova *et al.*, 2017; Mu *et al.*, 2017; Thomas *et al.*, 2016). Specifically, several *L. reuteri* strains have shown antimicrobial potential to pathogens (e.g., *Escherichia coli* CCM 3988 and *Pseudomonas aeruginosa* CCM 3955) by producing organic acids, reuterin, ethanol, etc. (Greifova *et al.*, 2017). In mouse of acute colitis, *L. reuteri* ATCC PTA 6475 alleviated inflammation, and a specific gene related to immunomodulation was identified (Thomas *et al.*, 2016). Similarly, colitis severity of mice was reduced by two *L. reuteri* strains potentially due to a tightened epithelial junction and thicker mucous layer (Ahl *et al.*, 2016). Even though a collection of strains has been commercialized as probiotics, it is starting to draw more attention to choosing appropriate candidates for probiotics. For instance, studies have revealed that most benefits exerted by *L. reuteri* are strain-specific (Su *et al.*, 2017; Yang *et al.*, 2015b). Furthermore, considering the decreased abundance of *L. reuteri* in the human gut, it will be exceedingly fascinating to achieve the reintroduction of the species.

1.4.3 *L. reuteri* as a model organism

Due to host-specificity in *L. reuteri*, as mentioned above, this bacterial species has been established as a model organism to look into mechanisms by which microbes co-evolve with their

hosts (Duar *et al.*, 2017; Kwong and Moran, 2015; Oh *et al.*, 2010). Additionally, *L. reuteri* was widely used in studies about the role of colonization history in the gut microbiota variation (Martinez *et al.*, 2018) and cross-feeding among microbial members in the GI tract (Cheng *et al.*, unpublished data). Most studies of this field were conducted in mouse models (Frese *et al.*, 2013; Krumbeck *et al.*, 2016), sometimes in chicken (Duar *et al.*, 2017) and swine (Wang *et al.*, 2008), but are largely lacking in other animals. For humans, studies on *L. reuteri* are difficult because of the low abundance of this species in the intestine. However, this comes with the advantage to use humans in studies on this bacterial species without the obstruction of background *L. reuteri*.

1.5 Critical need

Improved absolute quantification methods are needed in the field of gut microbiota research because current sequencing techniques are only semi-quantitative with limited sensitivity. Absolute quantification is necessary to determine how specific species or strain associate with health outcomes in human studies (Ferrario *et al.*, 2014; Maldonado-Gomez *et al.*, 2016; Zmora *et al.*, 2018), where persistence after probiotic administration of strains is often low. Therefore, improved limit of detection and accuracy of quantification method is needed. Similarly, for clinical diagnosis, high sensitivity is crucially desired as potentially pathogenic bacteria are often present at low concentrations in samples.

L. reuteri, as a member of the gut microbiota, can be used as a model organism. Quantification of *L. reuteri* can be achieved by cultivation based on selective media. A previous study has presented a *L. reuteri* specified medium (LRIM) modified from the Rogosa medium, which was designed for isolation, enumeration of lactobacilli. By removing all carbon sources but raffinose, it was claimed to be sufficiently selective for this organism in the presence of numerous

microbes from feces (Duar *et al.*, 2017). However, this method is extremely time-consuming and relies highly on the vitality of bacterial cells.

PCR-based technology is preferred, which is more specific and less laborious compared to culture. For the past two decades, the most widespread method for has been qPCR, which provides rapid and reliable quantification of *L. reuteri* with the limit of detection between 10^4 and 10^6 CFU/g or gene copies/g (Dommels *et al.*, 2009; Rattanaprasert *et al.*, 2014; Yang *et al.*, 2015a). Recently, the next-generation PCR (ddPCR) has claimed superiority to qPCR in aspects of sensitivity, reproducibility, and tolerance to inhibitors. Even though there are limited studies related to microbial quantification in human stool using ddPCR (Gobert *et al.*, 2018; Yang *et al.*, 2014), the potential of handling samples with a complicated background in ddPCR was presented above; therefore, a promising approach for the absolute quantification of gut microbes in stool samples. Several studies have been conducted to compare qPCR and ddPCR, but the results were not consistent due to differences in sample source, DNA extraction method or standard-setting (Gobert *et al.*, 2018; Verhaegen *et al.*, 2016; Yang *et al.*, 2014), and thus it is not conclusive which technology outperforms the other.

Given that DNA is the starting material of PCR, the performance of PCR-based analyses is highly dependent on DNA extraction procedures. To date, there are a wide variety of DNA isolation methods available for microbial detection in feces, and studies have shown that extraction methods considerably impact PCR results (Fock-Chow-Tho *et al.*, 2017; Kaisar *et al.*, 2017; Nechvatal *et al.*, 2008). For the detection of *L. reuteri* in feces, a major difficulty is that *L. reuteri* is a gram-positive bacterium with complex cell walls, which are hard to break. A search of publications has revealed that some studies used commercial kits (Dommels *et al.*, 2009; Yang *et al.*, 2015a), while others have used phenol chloroform-based methods (Rattanaprasert *et al.*, 2014;

Martinez *et al.*, 2018). However, the sensitivity of detection, as mentioned above, varied considerably. Thus, it is pivotal to find out effective DNA purification methods compatible with downstream PCR analysis. Overall, a comprehensive comparison between two PCR systems based on DNA extracted from different protocols is lacking in the field of fecal microbe quantification.

1.6 Specific objectives

The overall objective of this study was to develop methods for the quantitative detection of specific bacterial species and strains in human fecal samples that were improved in sensitivity, accuracy, reproducibility, time, and cost. This objective was addressed using the following specific aims:

a. Compare three DNA extraction methods in terms of DNA quality and purity, and their impact on downstream PCR analyses. In this study, we used one phenol chloroform-based method which was widespread across labs, including us (Martinez *et al.*, 2009) and two QIAamp kit-based protocols suggested in publications (Costea *et al.*, 2017; Maksimov *et al.*, 2017).

b. Compare qPCR and ddPCR for the quantification of *L reuteri* in fecal samples. To achieve this goal, we stimulated the colonization of *L. reuteri* in the human gut through spiking different loads of DSM 17938 into fecal specimens free of *L. reuteri* (Dalla-Costa *et al.*, 2017; Leach *et al.*, 2018), and performed qPCR and ddPCR in parallel.

c. Apply the optimized methodology for the strain-specific detection of *L. reuteri* strains used in a human feeding trial. The two strains are PB-W1, one of the isolates we obtained from Papua New Guinea, and DSM 20016^{T}, the type strain of *L. reuteri*.

By conducting this study, we presented a useful tool for specific quantification of bacteria, including but not limited to *L. reuteri*, in fecal samples, and provided general guidance of method development.

2. Materials and methods

2.1 Bacterial strains and growth conditions

L. reuteri strains DSM 17938 was grown on MRS agar (BD Difco Microbiology, Houston, TX, USA) for 24 hours in an anaerobic chamber at 37 °C. Single colonies were picked and transferred to MRS broth (BD Difco Microbiology, Houston, TX, USA) and sub-cultured twice and grown first for 24 hours, and then to late exponential phase. Cell numbers in cultures were determined by plating a tenfold serial dilution on MRS agar.

2.2 Spiking of fecal samples

Fecal sample collection was completed at the University of Alberta in accordance to the ethics protocol Pro00077565 approved by The Health Research Ethics Board - Biomedical panel of the university of Alberta. To evaluate the performance of qPCR and ddPCR, *L. reuteri* strain DSM 17938 was used to spike fecal samples, and the absence of *L. reuteri* DSM 17938 was confirmed through qPCR. Serial dilutions of bacteria with exact cell numbers were prepared by ten-fold dilution, resulting in fecal samples ranging from$10^{7.97}$, $10^{6.97}$, $10^{5.97}$, $10^{4.97}$, $10^{4.67}$, $10^{4.37}$, $10^{4.07}$, $10^{3.77}$ CFU/g. After homogenization, all spiked samples were stored in a -80 °C freezer until DNA isolation.

2.3 DNA extraction

Three commonly used protocols designed for DNA extraction from fecal samples were compared: a phenol chloroform-based method (PC; Martinez *et al.*, 2009), a modified method using QIAamp Fast Stool DNA kit (QK; Maksimov *et al.*, 2017), a method based on the protocol Q (PQ; Costea *et al.*, 2017). The details of each method are described below. Average concentration of extracted DNA and standard deviation (SD) was calculated and presented in Mean ± SD. The reproducibility of each method was reflected by coefficient of variation (CV).

2.3.1 Procedures of PC

One gram of stool was diluted ten-fold in ice-cold PBS buffer (NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.44 g, KH_2PO_4 0.24 g, MilliQ Water 1 L, pH 7.0). The samples were vortexed vigorously, and 1 ml of the solution was transferred to a 2 ml centrifuge tube and washed three times with PBS buffer with centrifugation (8000 × g for 5 min at 4 °C) in between. After the initial washing step, fecal pellets were resuspended in 750 µl lysis buffer (200 mM NaCl, 100 mM Tris [pH 8.0], 20 mM EDTA, and 20 mg/ml lysozyme). The suspensions were transferred to a 2 ml sterile tube containing 0.3 g Zirconia beads (0.1 mm, BioSpec Products Inc., Bartlesville, OK, USA) and incubated at 37 °C for 20 min. After 85 µl of 10% SDS solution and 30 µl proteinase K (20 mg/ml) were added, the mixtures were incubated at 60 °C for 30 min. Next, 500 µl of phenol-chloroform-isoamyl alcohol (25:24:1) was added, and the samples were homogenized in a FastPrep cell disrupter (MP Biomedicals, LLC, USA) at maximum speed for 2 min. Solutions were placed on ice and centrifuged at 10,000 × g for 5 min. Aqueous phases were transferred carefully to a new 1.5 ml microcentrifuge tube, followed by two extractions with phenol-chloroform-isoamyl alcohol (25:24:1) and two extractions with Chloroform-Isoamyl alcohol (24:1). DNA was

precipitated with anhydrous ethanol and 3 M sodium acetate and dissolved in 100 µl of Tris-Buffer (10 mM, pH 8.0).

2.3.2 Procedures of QK

This method was adapted from a previous publication (Maksimov *et al.*, 2017) with some changes. The initial washing of fecal samples in ice-cold PBS was conducted following steps mentioned in **section 2.3.1**. Next, DNA was extracted from 0.1 g stool using QIAamp Fast DNA Stool Mini Kit (Qiagen) with initial enzymatic lysis and bead-beating steps. Briefly, 100 µl of lysis buffer (200 mM NaCl, 100 mM Tris [pH 8.0], 20 mM EDTA, and 20 mg/ml lysozyme) was added and mixed well with fecal pellets by vortexing. Next, suspensions were incubated at 37 °C for 30 min and treated with 1 ml of buffer InhibitEX. Samples were homogenized thoroughly by vortexing and transferred to a tube containing 0.3 g of zirconium beads (0.1 mm, BioSpec Products Inc., Bartlesville, OK, USA). After the process of bead beating, DNA was isolated following the standard protocol of the kit.

2.3.3 Procedures of PQ

The procedure was conducted following the protocol from (Costea *et al.*, 2017) with a few modifications. Similarly, fecal samples were firstly washed in ice-cold PBS, as presented in **section 2.3.1**. One hundred milligrams of frozen feces were mixed with 1.0 ml ASL lysis buffer (Qiagen) by vortexing for 2 min in a 2 ml tube containing 0.3 g of sterile zirconia beads (0.1 mm, BioSpec Products Inc., Bartlesville, OK, USA). Samples were heated for 15 min at 95 °C and mechanically lysed by running the Fastprep™ Instrument (MP Biomedicals, LLC, USA) for 2 min. Samples are allowed to cool down on ice for 2 min, and then centrifuged at 16000 × g at 4 °C for

five minutes. Supernatant was transferred to a new 2 ml tube. The pellet was mixed with 300 µl ASL lysis buffer, and steps of heating and bead beating were repeated. The two supernatants resulting from the two extraction steps were pooled in a new 2 ml tube, and 260 µl of 10 M ammonium acetate was added to each lysate tube, mixed well, and incubated on ice for 5 min. Then, the mixtures were centrifuged at 16000 × g at 4 °C for 10 min. Supernatants were aliquoted to two 1.5 ml tubes with the addition of one volume of isopropanol, homogenized well, and stored at -20 °C overnight. DNA pellets were recovered by centrifuging at 16000 × g at 4 °C for 15 min. Pellets were washed with 70% ethanol (0.5 ml) and dried at room temperature for one hour. Next, the nucleic acid pellets were dissolved in 100 µl of TE (Tris-EDTA) buffer, and the two aliquots were pooled. The purification of DNA was carried out following the standard protocol of QIAamp Fast DNA Stool Mini Kit.

2.4 Quantification of *L. reuteri* DSM 17938 in fecal samples

To comprehensively compare qPCR and ddPCR, cell numbers of DSM 17938 were measured by both methods using strain-specific primers developed in a previous study (Egervarn *et al.*, 2010). This primer pair (1694f: 5′-TTAAGGATGCAAACCCGAAC-3′ and 1694r: 5′-CCTTGTCACCTGGAACCACT-3′) targets a chromosome-located surface protein gene Ir1694 which has single copy on the genome, and the amplicon length is 177 bp (Egervarn *et al.*, 2010).

2.4.1 ddPCR

ddPCR was performed using EvaGreen intercalating DNA dye to detect positive droplets. Each ddPCR reaction contained 1 µl of DNA (DNA extracted using PC and PQ was treated with a 10-fold and 3-fold dilution, respectively), 12.5 µl of 2 × EvaGreen Supermix (Bio-Rad

Laboratories Inc., Hercules, CA, USA), 200 nM primer each, and ddH_2O to bring the per-reaction volume to 25 µl in each well of a 96-well plate. The plate was put into a QX200 Auto Droplet Generator (Bio-Rad Laboratories Inc.), after which EvaGreen droplet generation oil (Bio-Rad Laboratories Inc.) was added according to the manufacturer's manual. PCR reactions were conducted in a Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad Laboratories Inc.) as following: (1) 95 ℃ for 5 min, (2) 95 ℃ for 30 sec, (3) 62 ℃ for 1 min, (4) steps 2 and 3 repeated for 39 cycles, (5) 4 ℃ for 5 min, (6) 90 ℃ for 5 min, and (7) a hold at 4 ℃. After the thermal cycling, the plate was placed in the block of a Bio-Rad QX200 Droplet Reader (Bio-Rad Laboratories Inc.). Droplets were read one at a time, and data were analyzed in QuantaSoft Analysis Pro 1.0 (Bio-Rad Laboratories Inc.). Single well thresholding was used to group droplets through either the software's default internal algorithm or manual setting.

2.4.2 Standard curve for qPCR

A standard curve was constructed by serially dilution genomic DNA of *L. reuteri* strain DSM 17938. The copy number of the standard material was first calculated based on the DNA concentration determined spectrophotometrically using NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) and calibrated by ddPCR, ranged from 4×10^5 to 4 gene copies/µl (4×10^8 to 4×10^3 CFU/g feces).

2.4.3 qPCR

The reaction mixture consisted of 1 µl of DNA (DNA extracted using PC was treated with a 10-fold dilution), 2 µl of SYBR Green Master Mix (FroggaBio, #BIO-92005), 0.6 µl of each primer (10 µM), and 7.8 µl of nuclease-free water. The qPCR program was composed of an initial

denaturation step for 3 min at 95 ℃, followed by 40 cycles of denaturation for 5 sec at 95 ℃, annealing and extension for 30 sec at 62 ℃ and fluorescent signal acquisition. PCR amplification was achieved using an HT 7900 machine (Thermo Fisher Scientific, Waltham, MA, USA). After forty cycles of PCR amplification, a melting analysis was conducted to verify the correct product by its specific melting temperature Tm. The reaction program for melting curve analysis included a denaturation for 15 sec at 95 °C, lowered to 60 °C for 1 min, and increased to 95 °C for 15 sec with continuous fluorescence readings.

2.5 Quantification of *L. reuteri* PB-W1 and DSM 20016^T in fecal samples

2.5.1 Bacterial strains and growth conditions

L. reuteri PB-W1, DSM 20016^T and other strains originating from human (Cor124_1_1, Cor137_1_1, Cor137_3_1, LMS11-3, LMS11-1, SR11, SR14, ME-262, ME-261, MM36-1a, MV4-1a, M81-R43, FJ3, PB-W2, MM4-1a, DSM 17938, CF48-3A, MM34-4A, MM2-3) were grown and collected as described in **section 2.1**.

2.5.2 Strain/Lineage-specific primer design for PB-W1 and DSM 20016^T

L. reuteri PB-W1 and DSM 20016^T belong to different *L. reuteri* lineages (Li *et al*., unpublished data). Unique genes or sequences in these two lineages were identified by comparing their genomes with a selection of human strains from other lineages in the JGI database (https://jgi.doe.gov/) and a local software Roary (find details in **Fig. 1** and **Fig. 2**). From this analysis, genes encoding for Lactococcin A secretion protein LcnD in PB-W1 and a hypothetical protein in DSM 20016^T were selected for primer design. Primer sets were designed by Primer3Plus

(https://primer3plus.com/cgi-bin/dev/primer3plus.cgi) with *in silico* validation (**Fig. 3**). Sequences and amplicon size of primers are listed in **Table 1**.

To experimentally validate the specificity of these designed primers, genomic DNA from other representative human strains was prepared. Furthermore, fecal samples of nine healthy human subjects were collected as part of the project 'Rewilding the Human Gut: Reintroduction of the Species *Lactobacillus reuteri*' (https://clinicaltrials.gov/ct2/show/NCT03501082), which was approved by the IRB board of the University of Alberta under protocol number Pro00077565. DNA isolation for these fecal samples was conducted using method PQ (see **section 2.3.3** for details), and then qPCR using these two pairs of primers was conducted against these DNA samples.

2.5.3 Spiking of fecal samples

The spiking of *L. reuteri* PB-W1 and DSM 20016^{T} were carried out following the same procedure mentioned in **section 2.2**, with specific bacterial concentrations ($10^{8.34}$, $10^{7.34}$, $10^{6.34}$, $10^{5.34}$, $10^{4.95}$, $10^{4.55}$, $10^{4.15}$, $10^{3.75}$, $10^{3.35}$, $10^{2.96}$ CFU/g feces for PB-W1, and $10^{8.01}$, $10^{7.01}$, $10^{6.01}$, $10^{5.01}$, $10^{4.01}$, $10^{3.61}$, $10^{3.21}$, $10^{2.81}$ CFU/g feces for DSM 20016^{T}).

2.5.4 Quantitative detection using qPCR

After DNA extraction by method PQ (presented in **section 2.3.3**), cell numbers of *L. reuteri* PB-W1 and DSM 20016^{T} were determined using qPCR with strain/lineage-specific primers designed in **section 2.5.2**. The protocol was generally identical to the one mentioned above in **section 2.4.3** with minor modifications. Firstly, 0.8 µl of each primer (10 µM) was added, while the total volume of each reaction remained unchanged. Secondly, PCR cycling was changed: initial

denaturation for 3 min at 95 ℃, followed by 40 cycles of denaturation at 95 ℃ for 5 sec, annealing at 63 ℃ for 10 sec, and extension at 72 ℃ for 15 sec. Lastly, standards were established with DNA extracted from a pure culture of each strain. Instead of using a spectrophotometer as described in **section 2.4.2**, the quantity of standard was matched with the actual bacteria load determined by quantitative plating. A standard was strictly required on every plate.

2.6 Statistical analysis

Values in Table 2 and Table 3 are shown as mean ± SD. Statistical tests were performed using R 3.4.2 (R Core Team, 2014). R^2 and equations of linear regression in Fig. 4, Fig. 7, Fig. 8, and Fig. 9 were calculated using R 3.4.2.

3. Results

3.1 Yield and quality of extracted nucleic acids

The color of DNA solutions obtained from method QK and PQ were visually clear, while it varied from pale yellow to brown for method PC. DNA concentrations varied substantially depending on methods used. As shown in **Table 2**, method PC generated DNA of highest concentration (1525.31 ± 290.24 ng/μl), followed by method PQ (694.34 ± 76.17 ng/μl) and QK (259.29 ± 40.80 ng/μl). Spiking of *L. reuteri* cells did not have a prominent effect on the total DNA yield because spiked bacteria only accounted for a small proportion of total fecal microbes (data not shown).

The purity of DNA was also determined spectrophotometrically for all samples, and two ratios ($A_{260/280\ nm}$ and $A_{260/230\ nm}$) were compared. All DNA solutions showed excellent $A_{260/280nm}$ values (1.95 - 2.12) with method PQ being slightly higher, indicating a more efficient removal of

protein from DNA. However, $A_{260/230\ nm}$ values indicated that QK and PQ resulted in DNA with less contamination of phenol, carbohydrate and humic acid ($A_{260/230\ nm}$ = 1.99 ± 0.15 for QK and 2.10 ± 0.15 for PQ), while DNA solutions obtained by method PC showed notably lower values (1.74 ± 0.16). Overall, although all three DNA isolation methods produced nucleic acids with acceptable quantity and quality, DNA isolated with methods QK and PQ was of higher purity.

3.2 Standard curve for qPCR

To evaluate the performance of qPCR and ddPCR for the quantification of *L. reuteri* in fecal samples in aspects of sensitivity, reproducibility, and accuracy, fecal samples from three human subjects were spiked with a series of diluted *L. reuteri* cultures, which were analyzed using qPCR and ddPCR in parallel. For the qPCR assay, a 10-fold serial dilution of genomic DNA of DSM 17938 was used to construct the standard curve (see **section 2.4.2**). To avoid the bias caused by spectrophotometer, the DNA was further measured by ddPCR. According to the outcomes of ddPCR, it turned out that the spectrophotometric measurement did overestimate the DNA concentration, and thus the concentration of reference materials was corrected. All qPCR standard curves showed acceptable linearity ($R^2 > 0.99$) and efficiency (**Fig. 8** (D)). The generation of the standard curve also demonstrated that the qPCR system covered DNA concentrations from at least six orders of magnitude.

3.3 Comparison of linearity, limit of detection (LOD), limit of quantification (LOQ), and reproducibility between qPCR and ddPCR

For both the qPCR and ddPCR approaches, data for gene copies per reaction were transformed to absolute numbers of cell forming units per gram feces following the formula ($C \times$

V_T)/($V_U \times M$), where C is gene copies measured per reaction, V_T is the elution volume of extracted DNA, V_U is the volume of DNA used, and M is the amount of fecal sample used in DNA extraction. As shown in **Fig. 4** (A-C), the measurements of qPCR in combination with the two kit-based DNA isolation procedures exhibited good linearity through the whole dilution series (R^2 = 0.9801 and 0.9927 for method QK and PQ respectively), while method PC showed relatively lower level of linearity with R^2 being 0.9533. Similarly, ddPCR results (**Fig. 4** (D-F)) showed better linearity with method QK (R^2 = 0.9926) and PQ (R^2 = 0.9906) compared to method PC (R^2 = 0.9709). Overall, even though ddPCR outperformed qPCR slightly with method QK and PC in terms of the linearity, both methods generated comparable and good results when methods QK and PQ were used.

Since the qPCR measurement in the study was conducted without a probe, the detection of the correct target sequence was confirmed by a melting curve analysis. Melting temperature (Tm) of the specific amplicon from the genome of *L. reuteri* DSM 17938 was approximately 87.9 ℃ (**Fig. 5**), which means peaks at other temperatures represent non-specific amplification or primer dimers.

As shown in **Table 3**, the *L. reuteri* DNA could still be detected at the input of 3.77 Log_{10} CFU/g feces in the qPCR analysis with DNA extracted using methods QK and PQ, while the capability of detection ended at the input of 4.67 Log_{10} CFU/g feces for method PC, which may be caused by the 10-fold dilution applied to DNA isolated. Undiluted DNA obtained with PC did not result in successful PCR reactions, making dilutions necessary. The LODs of qPCR are 3.95 Log_{10}, 4.86 Log_{10}, and 4.11 Log_{10} CFU/g feces for QK, PC, and PQ (**Table 3**).

To evaluate the ability of two quantitative systems to reliably quantify *L. reuteri* in fecal samples, the variation among replicates were compared. ddPCR had a better reproducibility as

values of CV were consistently lower compared to qPCR (**Table 3**). However, this was not as noticeable when method PQ was used for DNA extraction. The LOQ was calculated following the formula ($M_B + 10 \times S_B$) as suggested by American Chemical Society guidelines (1980), where M_B is mean of non-spiked fecal samples (blank), and S_B is the standard deviation of non-spiked fecal samples (blank). After calculation, the LOQs are 4.65 Log_{10}, 5.81 Log_{10}, and 4.50 Log_{10} CFU/g feces for qPCR and 4.30 Log_{10}, 5.09 Log_{10}, and 4.70 Log_{10} CFU/g feces for ddPCR with method QK, PC, and PQ respectively. Since there was no robust differentiation between LOD and LOQ in terms of non-specific amplification for ddPCR with Evagreen. We determined to evaluate LOD and LOQ separately for qPCR and only apply LOQ for ddPCR. Generally, ddPCR showed a lower LOQ than qPCR, especially when PC was used for DNA extraction (5.09 Log_{10} vs. 5.81 Log_{10} CFU/g feces). However, better performance of quantification was still observed in both qPCR and ddPCR when methods QK and PQ was used for DNA isolation; therefore, method PC was excluded from further analyses to focus more on applicable and suitable approaches.

3.4 Comparison of accuracy between qPCR and ddPCR assays

To assess the accuracy of qPCR and ddPCR with DNA purification methods QK and PQ, we determined the recovery rate of *L. reuteri* in the detection as suggested in a previous study (Wang *et al.*, 2018). This measurement reflects the percentage of cells detected out of the theoretical spiking concentration. As shown in **Fig. 6**, method PQ resulted in higher recovery rates in both PCR methods compared to method QK, indicating a higher efficiency of method PQ in the recovery of DNA from *L. reuteri* DSM 17938. Compared to ddPCR, higher values of recoveries were also observed in qPCR. However, the cell number was relatively overestimated at the input of 3.77 Log_{10} CFU/g feces. This overestimation was probably caused by a difference in

amplification efficiency between standard and fecal samples. Nevertheless, the discrepancy within detectable range was not larger than 0.34 after transforming into Log_{10} values, this bias is thus negligible when quantifying gut bacteria in fecal samples. It is worth noting that ddPCR stably produced recoveries between 73.2% and 83.1% with method PQ within the quantitative range, which is superior to qPCR.

3.5 Correlation between qPCR and ddPCR

The qPCR and ddPCR measurements in combination with method PQ for spiked fecal samples were compared (**Fig. 7**). Excellent correlation was obtained between these two techniques ($y = 1.0607x - 0.4746$, $R^2 = 0.9913$) with qPCR generally showing higher cell numbers (0.12 ± 0.11 Log_{10} CFU/g) than ddPCR.

3.6 Comparison of costs between qPCR and ddPCR

The estimation of cost for either qPCR or ddPCR was calculated per 96-well plate (Applied Biosystems), which reduces the costs of consumables per sample as compared to lower numbers of samples that would be analyzed by a single PCR run. Assuming that all samples, standard materials, and controls are run in triplicate, the maximum number of samples that can be analyzed per plate are 25 for qPCR and 30 for ddPCR. As shown in **Table 4**, the total cost per 96-well plate for ddPCR was almost four times as high as that for qPCR (12.5 CAD vs. 3.2 CAD). The time spent conducting ddPCR (6.5 h) was also increased when compared to qPCR (2.5 h). Therefore, if take the payment on labor into consideration, the cost of both methods will be increased but more prominently for ddPCR.

3.7 Development of a qPCR system for the accurate quantification of *L. reuteri* PB-W1 and DSM 20016^T

3.7.1 Strain/lineage-specific primers

Primers were designed based on unique genomic genes/sequences identified by genome comparison using phylogenetic profiler on the JGI website or a local software Roary. A gene encoding Lactococcin A secretion protein LcnD for PB-W1 and a genomic sequence encoding hypothetical protein for DSM 20016^T was found to be unique to at least the phylogenetic lineages.

Primer pairs were tested for specificity using genomic DNA from the target strains and a set of representative *L. reuteri* strains. The optimal annealing temperature for both sets of primers was determined to be 63 ℃ based on the efficiency of PCR reactions, at which amplification efficiency was maintained acceptable when non-specific signals was kept at the lowest level. The specificity of primers was evaluated under the optimized conditions against representative human strains. Most strains other than those for which the primers were designed showed no amplification (**Table 5**). Three non-target strains (Cor137_1_1, Cor137_3_1, and MV4-1a) showed amplification with the primers for PB-W1. However, Cq values were over 35, which was high when compared to the signal obtained with PB-W1 (Cq = 10). For the primer system for DSM 20016^T, four strains resulted in positive readings (Cor137_1_1, Cor137_3_1, MM36-1a, and CF48-3A). However, amplification only occurred after 33 cycles compared to 9.5 cycles for DSM 20016^T. The difference of Cq values between target and non-target strains was at least 25 and 23.5 cycles for PB-W1 and DSM 20016^T, which accounts for a difference of more than 10^7 times. This result showed that with the same amount of DNA, non-target strains would only have a negligible impact on the accurate quantification of target strains. Non-specific amplification can be detected by performing a melt curve analysis, in which different strains produce melt curves with different

peaks. In the PB-W1 assay, the formation of non-specific products in Cor137_1_1 and Cor137_3_1 were not reproducible between technical replicates and showed distinct melting temperatures, which can be easily distinguished from the target strain. For DSM 20016^T, non-specific amplification of *L. reuteri* Cor137_3_1 showed a different melting temperature from the target, and the other three strains with non-specific reaction exhibited no reproducibility between technical replicates. Those strains (MV4-1a, Cor137_1_1, MM36-1a, and CF48-3A) that cannot be distinguished from the target by melt temperature all had considerably higher Cq values compared with PB-W1 or DSM 20016^T under the same DNA concentration (50 ng/µl), which will therefore have minimal impact on detection. Additionally, test on random fecal samples also showed good specificity. No non-specific amplification was observed with primers for PB-W1, while fecal samples from three subjects exhibited positive readings using primers for DSM 20016^T. Two of them were under the LOQ, however, there was one stool sample harboring quantifiable bacteria. After isolation and culturing, the strain was identified as *L. reuteri* by 16S rRNA gene sequencing (data not shown), but whether the strain clusters with DSM 20016^T in the same lineage remains to be determined.

3.7.2 Quantification of PB-W1 and DSM 20016^T cells in artificially spiked fecal samples using qPCR

We spiked fecal samples with *L. reuteri* strains to obtain cell numbers from 2.96 Log_{10} to 8.34 Log_{10} CFU/g feces for PB-W1, and from 2.81 Log_{10} to 8.01 Log_{10} CFU/g feces for DSM 20016^T. After the isolation of DNA with method PQ, cell numbers of both strains were determined using qPCR with strain/lineage-specific primers. Standard curves were established with serial dilutions of bacterial genomic DNA extracted from the same cultures that were used for spiking.

The amplification efficiency was 94.2% for PB-W1 and 93.7% for DSM 20016^T, indicating good quality of PCR reactions. The analysis revealed a range for both PCR systems where correlations between the number of bacterial cells added to fecal samples and those that were quantified by qPCR was remarkable (**Fig. 9**). For PB-W1, this range was between 3.05 Log_{10} and at least 8.15 Log_{10} CFU/g feces (R^2 = 0.9994), while for DSM 20016^T, it was slightly narrower ranging from 2.96 Log_{10} and at least 7.74 Log_{10} CFU/g feces (R^2 = 0.9947). Within this range, the reproducibility of three independent tests was also good (**Table 6**). However, when the number of bacteria spiked was 2.96 CFU/g feces for PB-W1, the detection lost linearity, demonstrating the LOD.

4. Discussion

Driven by the need to develop a powerful tool for the reliable detection of bacterial strains in fecal samples with higher sensitivity and accuracy, we performed the present study. Within our best knowledge, this is the first study to comprehensively compare qPCR and ddPCR for the quantification of *L. reuteri* in human stools at strain level based on three commonly applied DNA extraction methods for fecal samples (i.e., two kit-based [QK and PQ] and a phenol chloroform-based method [PC]).

The three DNA extraction methods we tested in this study are commonly used for the preparation of nucleic acids from stool samples for downstream molecular analyses, including whole genome shotgun sequencing, 16S rRNA gene sequencing and qPCR (Gobert *et al.*, 2018; Ranjan *et al.*, 2016). In light of previous studies, we made a few modifications to these protocols. Firstly, washing fecal samples with ice-cold PBS was carried out as reported previously (Maldonado-Gomez *et al.*, 2016; Martinez *et al.*, 2009; Walter *et al.*, 2005), which has been shown to facilitate the removal of potential PCR inhibitors partially. Secondly, it is well known that

lysozyme is effective to lyse the cell walls of gram-positive bacteria. However, lactobacilli appeared not to be significantly sensitive to its lytic activity, and response to lysis is likely to vary among strains (Xanthopoulos *et al.*, 2000). Nevertheless, as suggested in many studies (Egervarn *et al.*, 2010; Mackenzie *et al.*, 2010; Martinez *et al.*, 2015; Ortiz *et al.*, 2017), we decided to use lysozyme for our study. Lastly, the processing with bead beating was performed in all methods to enhance the disruption and detection of gram-positive bacteria in the human gut microbiota, as recommended in previous studies (Costea *et al.*, 2017; Santiago *et al.*, 2014).

Our results demonstrated that method PQ was a robust protocol for DNA extraction from stool samples with high yield and purity, which was in agreement with a previous study (Costea *et al.*, 2017). Method QK was also shown to produce DNA with decent quality and purity but lower concentration. On the other hand, PC harvested the highest amount of DNA, which was comparable with other investigators' reports (Salonen *et al.*, 2010; Yuan *et al.*, 2012); however, other parameters of this DNA in terms of quality and purity were much lower when compared to the other methods. This might be due to a large amount of DNA extracted from the background and the insufficient removal of co-extracted PCR inhibitors. Results obtained in this study cohered with a previous finding that method PC increased total DNA yield by 6 and 2 times on average compared to method QK and PQ but provided the least satisfying quantitative results in either qPCR or ddPCR with reduced sensitivity and accuracy (Yuan *et al.*, 2012). In terms of purity, technology using silica columns (QK and PQ) did not require hazardous chemicals (e.g., phenol and chloroform) with the membrane substantially enhancing separation of DNA and potential PCR inhibitions (Yang *et al.*, 1998). In the present study, this was reflected by a higher ratio of 260/230 nm in QK and PQ than in PC. In summary, the prototype of method PQ has proven to be excellent for human stool processing in metagenomic studies (Costea *et al.*, 2017). We adapted this protocol

with minor modifications to our study and found it suitable for PCR-based detections as well. Based on the findings described above, method PQ can be promising for the quantitative measurement of bacteria, especially gram-positive ones, in human fecal samples. However, the major limitation is that PQ is time-consuming and complicated owing to multiple steps of DNA precipitation and redissolution. The time spent on DNA isolation was doubled using PQ when compared to QK, which also produced PCR-quality DNA. This makes QK more suitable for high-throughput analysis of gut microbiota. As for choosing protocol PC, one should be more careful because it was not as good as the other two methods based on our PCR results. This did not cohere with a previous study where they used method PC and successfully detected a *Bifidobacterium longum* strain in human stool at around 4 Log_{10} cells/g by qPCR (Maldonado-Gomez *et al.*, 2016). We speculated this disagreement was a result from the different primer and probe system they used as well as the distinct way in which LOD was determined.

qPCR has a major limitation associated with its dependence on an external standard curve, which likely introduces biases. This stems from the challenge in achieving an accurate quantity of materials used as standard. In this study, we determined the amounts of nucleic acids in reference materials using a spectrophotometric instrument, after which gene copies were corrected by ddPCR, as recommended (Cao *et al.*, 2015; Yang *et al.*, 2014). Our qPCR results proved the practicability and robustness of this method, but there are always arguments about the way in which standards should be established. Strategy based on spectrophotometric measurement directly obtains the concentration of nucleic acids by measuring UV absorbance at a certain wavelength, which is easy and cost-saving. However, spectrophotometers will indistinguishably quantify compounds with absorbance at 260 nm, potentially resulting in an overestimation of DNA in reference materials. In our study, we observed a higher number of the gene copies determined

via spectrophotometry than ddPCR as well, which was in agreement with other studies (Cao *et al.*, 2015; Yang *et al.*, 2014). Currently, there is an alternative measurement based on fluorescent dyes such as EvaGreen and Picogreen, which specifically bind to double-stranded DNA. It should theoretically be more accurate than a spectrophotometry-based method and thus may be recommended in future studies regardless of the higher cost and longer handling time. Apart from the number of gene copies calculated based on DNA concentration, cell numbers quantified by plating, flow cytometry, and microscopy was also used as calibrator when applicable in some studies (Cao *et al.*, 2012; Frese *et al.*, 2012; Martinez *et al.*, 2018; Yang *et al.*, 2014). Additionally, there is possibly a difference in PCR efficiency between standard materials and different fecal samples, of which the influence is often underestimated. For example, a 10% difference in efficiency will lead to a fold change as large as 3.75 after 25 cycles of PCR reaction (Perez *et al.*, 2013). This can provide an explanation for the slight overestimation close to LOD by qPCR in the present study. It is worth mentioning that spiked sample-dependent standards were used in some studies (Ahlroos and Tynkkynen, 2009; Maruo *et al.*, 2006; Peng *et al.*, 2011). This strategy would be preferable for minimizing the influence of variation in the background and efficiency variations between different samples and standards. However, it loses practicability when samples with various backgrounds have to be analyzed due to difficulties in building individualized standard for each sample.

qPCR is often considered to be more susceptible to inhibitors than ddPCR, especially when implemented for environmental, rumen, and fecal samples containing inhibitors (e.g., fulvic and humic acid) as well as background DNA in large amount (Iker *et al.*, 2013). It can be expected because qPCR relies on amplification efficiency, which can be adversely affected by inhibitors, while ddPCR is hardly affected due to its virtue of being an end-point method (Pinheiro *et al.*,

2012). Nevertheless, our findings did not show any superiority of ddPCR on tolerance to inhibition. This was likely a result of DNA extraction with QK and PQ, guaranteeing the removal of potential PCR-inhibiting compounds and the reasonable performance of both qPCR and ddPCR.

Our findings demonstrated that ddPCR had the advantage of improved reproducibility over qPCR. Quantification via ddPCR is fulfilled by counting the number of positive droplets where anticipated amplification happens and the total number of droplets after thermal cycling. This binary attribute allows quantifications using ddPCR independent of standard curves, and PCR efficiency has little influence on the results of quantification. Our results showed that when biological replicates were measured, ddPCR presented a lower level of variability, which was consistent with literature in this regard (Hindson *et al.*, 2013; Stauber *et al.*, 2016; Yuan *et al.*, 2019). Collectively, ddPCR may improve inter-study comparability if conducted among different laboratories.

Our finding showed that ddPCR did not have prominently improved sensitivity when compared to qPCR. The LOQ of ddPCR were lower when compared to qPCR but only to a limited degree. The comparable sensitivity between qPCR and ddPCR was also observed in previous studies (Verhaegen *et al.*, 2016; Yang *et al.*, 2014). Interestingly, we observed variation in sensitivity within each PCR systems when different DNA extraction methods were applied in our study. The LOD and LOQ of qPCR were significantly affected by DNA preparation procedures with both QK and PQ providing good quantitative performance. The impact was not so profound for ddPCR, but QK can still be considered as a better choice for DNA preparation in the ddPCR assays. Therefore, we recommend looking at more DNA extraction methods when determining quantification strategies because they are as important as quantitative platforms.

Furthermore, there are several limitations in ddPCR assays in fecal sample analysis. Its ability to quantification highly depends on the difference in intensity of fluorescent signals between positive and negative partitions. The presence of background DNA at high concentrations often fail the separation of positive and negative droplets, especially in EvaGreen-based ddPCR measurement since this DNA intercalating dye binds to all double-stranded DNA including newly produced amplicons and background DNA. This problem might be solved by using a probe-based system, as described by (Strain *et al.*, 2013). With this approach, the authors succeeded in loading 1.5 µg of DNA into a 20 µl reaction without compromising the quantification. In the present study, we found that the separation of positive and negative droplets by fluorescent intensity became extremely difficult with a total DNA load of over 200 ng/reaction, and a dilution of template DNA was needed. Furthermore, ddPCR has a narrower dynamic range than qPCR since ddPCR is not able to detect more than 5 Log_{10} gene copies per reaction due to the limited number of droplets generated. It gives ddPCR limited flexibility when implemented in studies where samples of large microbial loads are detected. In the present study, qPCR and ddPCR showed a comparable detectable range, but had a different potential to cover a larger range. For example, with method PQ, ddPCR was nearly 80% saturated at the concentration of 7.97 Log_{10} CFU/g feces (data not shown), whereas for qPCR, the Cq value at the same amount of input was around 18 (**Fig. 8**). It is reasonable to expect the upper LOD of qPCR to reach 10 Log_{10} CFU/g feces, but ddPCR is theoretically not able to detect over 8 Log_{10} CFU/g feces, which will be a 2 Log_{10} difference. Besides, in the economic aspects, the utility of ddPCR is more expensive and labor-intensive compared with qPCR. Generally, based on assays developed in this study, the cost and time spent on a full-loaded 96-well plate in ddPCR are 4 times and 2.5 times more than qPCR.

Taken together, both QK and PQ provided DNA of high quantity and quality, with method PQ showing the most exceptional efficiency in covering *L. reuteri* genomic DNA from human fecal samples. ddPCR showed the lowest LOQ when QK was used, while both qPCR and ddPCR exhibited a high degree of linearity across the detectable range with the application of method PQ. In addition, qPCR is expected to a wider dynamic range, making it more applicable for a high throughput microbial detection in the stool. If taking time and cost under consideration, PQ and ddPCR are more time-consuming than QK and qPCR, and ddPCR is 4 times as expensive as qPCR. After all the assessments, we determined to use the combination of PQ and qPCR as the best for further studies.

We applied the optimized method identified during the first part of this thesis to develop strain/lineage-specific PCR assays for two *L. reuteri* strains, PB-W1 and DSM 20016^{T}, that are currently used in a human trial. Strains within a certain lineage are relatively close in aspects of genome and lifestyle (Oh *et al.*, 2010), so it can be impossible to identify genomic regions to distinguish strains. This applied especially for strains from the human/vertebrate lineage II due to its low genomic variation. Thus, we decided to design linage-specific primers for strain DSM 20016^{T} instead of strain-specific primers. Given that the presence of *L. reuteri* in the human GI tract is substantially rare (Martinez *et al.*, 2015; Walter *et al.*, 2001; Walter *et al.*, 2011), we believed our primers would perform as well as those designed for specific strains.

Our study demonstrated that it is possible to achieve successful quantification with strain/lineage-specific primers. Although they were not 100% specific if tested against non-target human strains, we merely observed a low level of false-positive amplification. The target was differed from non-target strains by at least 23.5 cycles, which would account for differences of 7 orders of magnitude in cell numbers. This background amplification is acceptable as *L. reuteri* is

only detected in human fecal samples at less than 9 Log_{10} per gram feces in previous studies (Duar *et al.*, 2017; Frese *et al.*, 2012; Rattanaprasert *et al.*, 2014).

In contrast to the first study, the standard curve for the strain/lineage-specific qPCR assays was based on cell numbers of *L. reuteri* determined on MRS plate and not gene copies. Our results confirmed that this method was fitting for the detection of specific strains in stool samples as well. However, the limitation is that only viable cells in reference materials would be taken into consideration, likely leading to underestimation of real samples. To reduce the percentage of dead cells, bacterial culture was harvested before reaching the stationary phase. The LOD showed in other studies varied from 5 Log_{10} CFU/g feces (Karjalainen *et al.*, 2012; Maruo *et al.*, 2006; Rattanaprasert *et al.*, 2014) to 4 Log_{10} CFU/g feces (Martinez *et al.*, 2018). Our study is the first to report successfully push the LOD under 4 Log_{10} CFU/g feces.

Although this study provided overall comprehensive comparison of two PCR-based measurements and three DNA isolation protocols for *L. reuteri* quantification in human fecal specimens, it is of importance to recognize certain limitations. Firstly, we only used three fecal samples with different backgrounds, with the variation between biological replicates being relatively large. In future studies, more samples can be included to obtain more accurate and robust results. Secondly, we did not test multiplex PCR with qPCR and ddPCR. In a scenario when one needs to detect more than one target in any sample, multiplex PCR will save time, materials, and labor costs if implemented properly. It has been reported that duplexing with qPCR was possible based on either double-stranded DNA intercalating dye (Asing *et al.*, 2016) or probe (Saulnier *et al.*, 2017). On the other hand, ddPCR was designed to run without standard and is not susceptible substrate competition because of individual reactions in micro-droplets. Multiplexing with ddPCR can be reliably achieved by size difference of product using DNA dye (Paquette *et al.*, 2018;

Weerakoon *et al.*, 2016) and fluorescence signal using probe (Cao *et al.*, 2015; Hamaguchi *et al.*, 2018). Furthermore, compared to qPCR, ddPCR is more advantageous in determining the ratio between several bacterial strains in a single reaction because of its independence of standard. For qPCR, calculation using independent standard curves for each target would potentially enlarge bias originated from differences in PCR efficiency and compromise the robustness of quantification.

This study provides guidance for the selection of DNA preparation methods and PCR techniques for an accurate absolute quantification of bacterial species and strains in human fecal samples. We tested three DNA extraction methods and two PCR systems in the first study and validated a good combination of qPCR and PQ in the second study, achieving excellent quantification of two *L. reuteri* strains in stool samples. Nevertheless, it neither means this strategy is perfect nor should other methods not be considered in future studies. For example, although we have shown that the use of QK and ddPCR even resulted in a lower LOQ, the use of ddPCR substantially increased costs and time of handling. Furthermore, qPCR outcomes can be validated by melting curve, resulting in lower LOD when compared to ddPCR. It may not be worthy to gain a slight improvement in LOQ for our purpose. However, for studies with multiple bacterial strains, ddPCR can be preferable as its independence of standards which facilitates absolute quantification of all strains in one reaction. Similarly, others may argue that it will take twice of the time to apply PQ than QK for DNA extraction (8 h vs. 4 h of hands on time for 10 samples), so QK is more applicable for large numbers of samples.

In addition to the systematic comparison and validation methods, outcomes of this thesis can be applied to future studies. By using the optimal DNA extraction method and quantification platform, the sensitivity was improved significantly, which improves our ability to connect gut

microbes to host biology when specific species or strains are present at low concentrations. For example, probiotics can exert beneficial effects when administered in adequate amounts (Hill *et al.*, 2014) but studies have claimed that most probiotic strains are not able to persist in the gut. It might be true based on current detection methods. However, this observation can be a result of limitations originated from detection tools. By using methods with higher sensitivity, we will be able to look at effects of probiotic present at lower concentration. Furthermore, it will expand our knowledge on the functionality of probiotics and composition of the gut microbiota. Similarly, for pathogen detection, higher sensitivity can be transferred to greater possibility of accurate diagnosis or even prevention of some diseases.

Tables

Table 1. Information of strain/lineage primers for *L. reuteri* PB-W1 and DSM 20016^{T}

Strain	Primer name	Nucleotide sequence (5' - 3')	Amplicon size
PB-W1	PLD-F PLD-R	TCGTGCTCCTAGAGATGGGA ACTTCTCCAGCTTTTACTGACGA	174 bp
DSM 20016^{T}	DHP-F DHP-R	GTGTTAAAGAGGTTGCTAGAAAGTATT GCCAGCTTAAATTCCTTTGAATAGC	139 bp

Table 2. Quantities and qualities of DNA extracted using different DNA isolation methods

	DNA concentration (ng/µl)	$A_{260/280\ nm}$	$A_{260/230\ nm}$
QIAamp Fast Stool DNA Kit (QK)			
Overall	259.29 ± 40.80	1.97 ± 0.02	1.99 ± 0.15
R1 (n=9)	256.62 ± 14.02	1.96 ± 0.01	1.90 ± 0.06
R2 (n=9)	218.67 ± 18.70	1.96 ± 0.01	2.16 ± 0.14
R3 (n=9)	302.59 ± 29.84	2.00 ± 0.01	1.91 ± 0.05
Phenol Chloroform (PC)			
Overall	1525.31 ± 290.24	2.00 ± 0.03	1.74 ± 0.16
R1 (n=9)	1391.42 ± 49.94	2.04 ± 0.01	1.92 ± 0.03
R2 (n=9)	1269.37 ± 30.06	1.97 ± 0.01	1.55 ± 0.03
R3 (n=9)	1915.14 ± 74.46	1.98 ± 0.01	1.76 ± 0.02
Protocol Q (PQ)			
Overall	694.34 ± 76.17	2.08 ± 0.03	2.10 ± 0.15
R1 (n=9)	737.92 ± 58.80	2.05 ± 0.02	2.00 ± 0.03
R2 (n=9)	615.07 ± 54.45	2.09 ± 0.02	2.31 ± 0.04
R3 (n=9)	730.03 ± 42.34	2.09 ± 0.01	2.00 ± 0.03

R1, R2, and R3 represent three fecal samples.

Table 3. Comparison of quantification performance of qPCR and ddPCR

Method	Log_{10} CFU/g feces Spiked	QIAamp Fast Stool DNA Kit (QK)		Phenol Chloroform (PC)		Protocol Q (PQ)	
		Data mean ± SD (Log_{10} CFU/g; n = 3)	CV (%)	Data mean ± SD (Log_{10} CFU/g; n = 3)	CV (%)	Data mean ± SD (Log_{10} CFU/g; n = 3)	CV (%)
qPCR	7.97	7.62 ± 0.13	1.77	7.53 ± 0.12	1.58	7.89 ± 0.12	1.53
	6.97	6.63 ± 0.14	2.12	6.53 ± 0.17	2.59	6.91 ± 0.11	1.55
	5.97	5.63 ± 0.21	3.66	5.61 ± 0.24	4.23	5.90 ± 0.04	0.60
	4.97	4.71 ± 0.16	3.44	4.92 ± 0.34	6.85	5.04 ± 0.11	2.20
	4.67	4.46 ± 0.18	3.96	4.86 ± 0.30	6.17	4.75 ± 0.10	2.18
	4.37	4.31 ± 0.16	3.67	-	-	4.54 ± 0.14	2.98
	4.07	4.08 ± 0.23	5.61	-	-	4.28 ± 0.07	1.67
	3.77	3.95 ± 0.17	4.22	-	-	4.11 ± 0.15	3.67
	Blank	3.72 ± 0.30	8.09	4.72 ± 0.49	10.38	3.89 ± 0.15	3.81
ddPCR	7.97	7.80 ± 0.03	0.34	7.43 ± 0.04	0.59	7.86 ± 0.08	1.07
	6.97	6.72 ± 0.02	0.30	6.41 ± 0.07	1.03	6.87 ± 0.11	1.66
	5.97	5.66 ± 0.04	0.74	5.40 ± 0.12	2.17	5.89 ± 0.11	1.79
	4.97	4.66 ± 0.05	1.10	4.88 ± 0.15	3.01	4.85 ± 0.09	1.92
	4.67	4.26 ± 0.16	3.69	4.76 ± 0.09	1.93	4.54 ± 0.05	1.17
	4.37	4.06 ± 0.05	1.25	-	-	4.29 ± 0.10	2.33
	4.07	3.74 ± 0.20	5.38	-	-	4.21 ± 0.23	5.41
	3.77	3.62 ± 0.21	5.91	-	-	3.86 ± 0.20	5.28
	Blank	3.57 ± 0.19	5.34	4.28 ± 0.22	5.20	3.66 ± 2.21	60.26

CV, coefficient of variation. The detection of target was verified by melting curves (Fig. 5). Dash, not detected. Blank, fecal samples without target strain.

Table 4. Estimate of cost per sample for detecting *L. reuteri* DSM 17938 in fecal samples, using either qPCR or ddPCR

Method	Specifications	Items and time	Cost	Cost per sample
qPCR	• A standard curve (6 dilutions, in triplicate) • 25 unknown samples and a no template control (all in triplicate)	reagents	CAD 60	CAD 3.2
		Consumables	CAD 20	
		2.5 h		
ddPCR	• A positive control (in triplicate) • 30 unknown samples and a no template control (all in triplicate)	Reagents	CAD 275	CAD 12.5
		Consumables	CAD 100	
		6.5 h		

Note: For qPCR, 25 samples (in triplicate) can be analyzed on a 96-well plate excluding no template controls. For ddPCR, 30 samples (in triplicate) can be analyzed on a 96-well plate excluding calibrators and controls. Cost for labor was not considered in this estimation.

Table 5. *L. reuteri* qPCR specificity test (Cq values and melting curve analysis) using non-target human strains from different lineages

*L. reuteri s*train	Source of origin	Lineage[a]	Cq mean	
			PB-W1	DSM 20016^T
PB-W1	human	VII	**10.0**	-
Cor124_1_1	human	VI	-	-
Cor137_1_1	human	VI	>35[b, d]	>34[d]
Cor137_3_1	human	VI	>36[b, d]	>34[c, d]
LMS11-3	human	II	-	NT
LMS11-1	human	II	-	NT
SR11	human	II	-	NT
SR14	human	II	-	NT
ME-262	human	II	-	NT
ME-261	human	II	-	NT
MM36-1a	human	VI	-	>34
MV4-1a	human	VI	>37[d]	-
M81-R43	human	VI	-	-
FJ3	human	II	-	NT
PB-W2	human	VII	-	-
DSM 20016^T	human	II	-	**9.5**
MM4-1a	human	II	-	NT
DSM 17938	human	VI	-	-
CF48-3A	human	VI	-	>33[d]
MM34-4A	human	VI	-	-
MM2-3	human	II	-	NT

NT, not tested because from the same lineage. Values in bold represents Cq values under the sample DNA concentration for PB-W1 and DSM 20016^T. Dash, no amplification.

[a] Data were obtained in publications (Li *et al*., unpublished data; Oh *et al.*, 2010).

[b] Melt curve showed different peak from PB-W1.

[c] Melt curve showed different peak from DSM 20016^T.

[d] Not reproducible between replicates.

Table 6. Performance of qPCR for the quantification of PB-W1 and DSM 20016^T in human fecal samples

PB-W1			DSM 20016^T		
Log_{10} CFU/g feces spiked	Mean Log_{10} CFU/g feces tested (n = 3)	CV (%)	Log_{10} CFU/g feces spiked	Mean Log_{10} CFU/g feces tested (n = 3)	CV (%)
8.34	8.15 ± 0.06	0.69	8.01	7.74 ± 0.04	0.54
7.34	7.19 ± 0.09	1.24	7.01	6.71 ± 0.07	1.00
6.34	6.15 ± 0.06	1.03	6.01	5.61 ± 0.05	0.91
5.34	5.17 ± 0.09	1.70	5.01	4.50 ± 0.07	1.60
4.95	4.68 ± 0.09	1.88	4.01	3.80 ± 0.11	2.96
4.55	4.35 ± 0.09	2.06	3.61	3.59 ± 0.18	5.09
4.15	3.98 ± 0.14	3.59	3.21	3.25 ± 0.15	4.53
3.75	3.53 ± 0.13	3.60	2.81	2.96 ± 0.21	7.17
3.35	3.05 ± 0.12	3.97			
2.96	3.08 ± 0.34	11.10			

Values with underline indicate LOD.

Figures

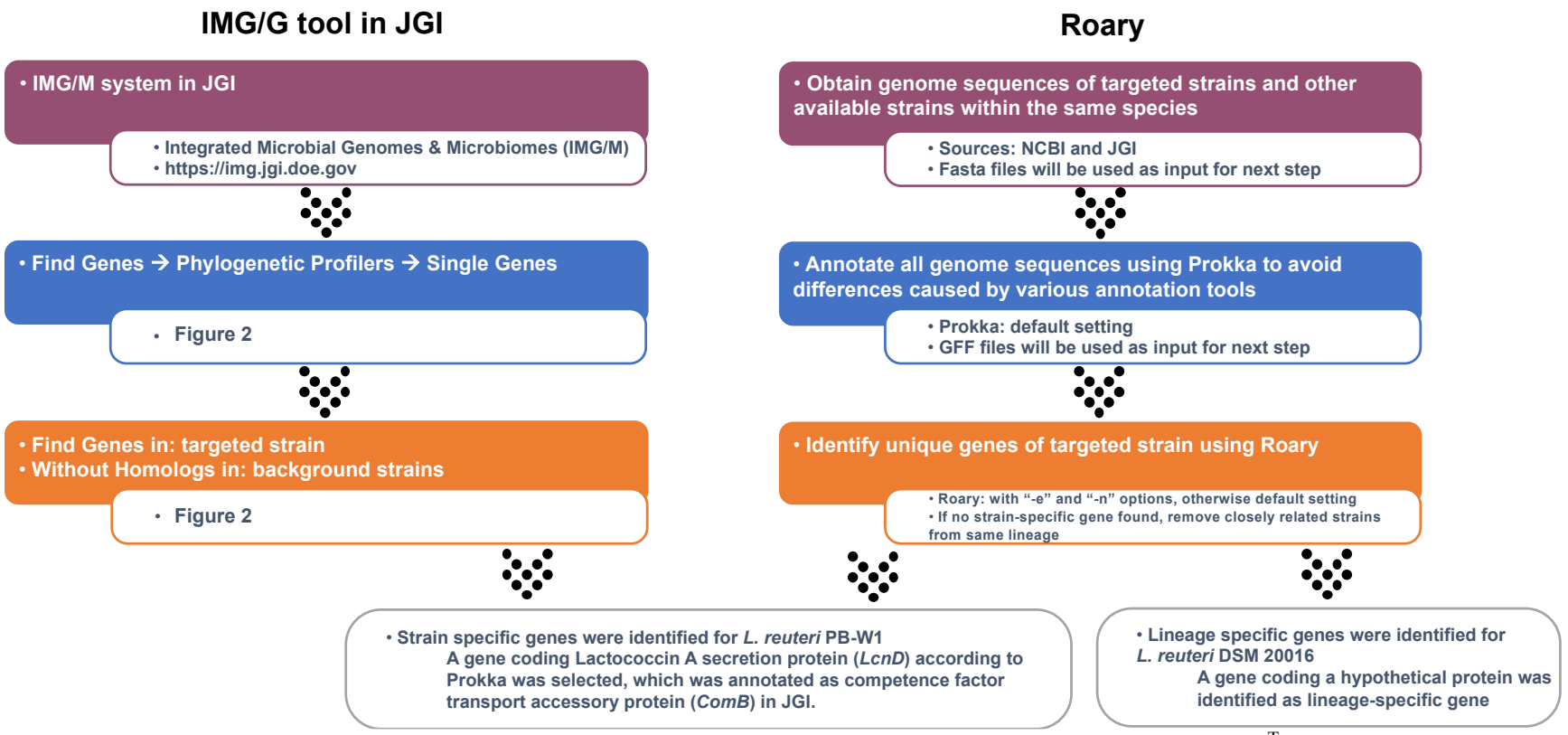


Figure 1. Flow chart of the identification of unique genes or sequences in *L. reuteri* PB-W1 and DSM 20016^{T}.

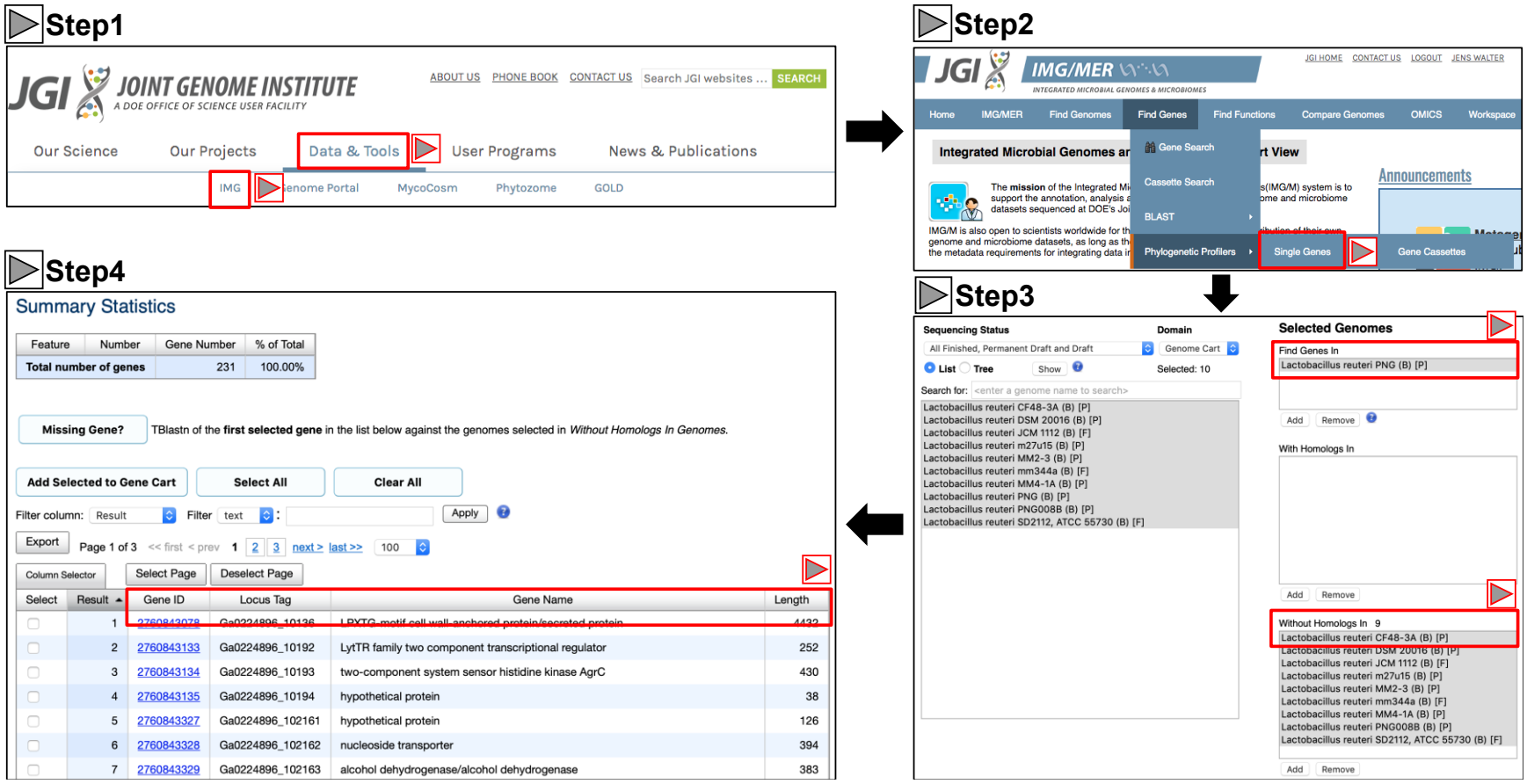


Figure 2. Identify unique genes of targeted strain using the IMG/M system provided in JGI.

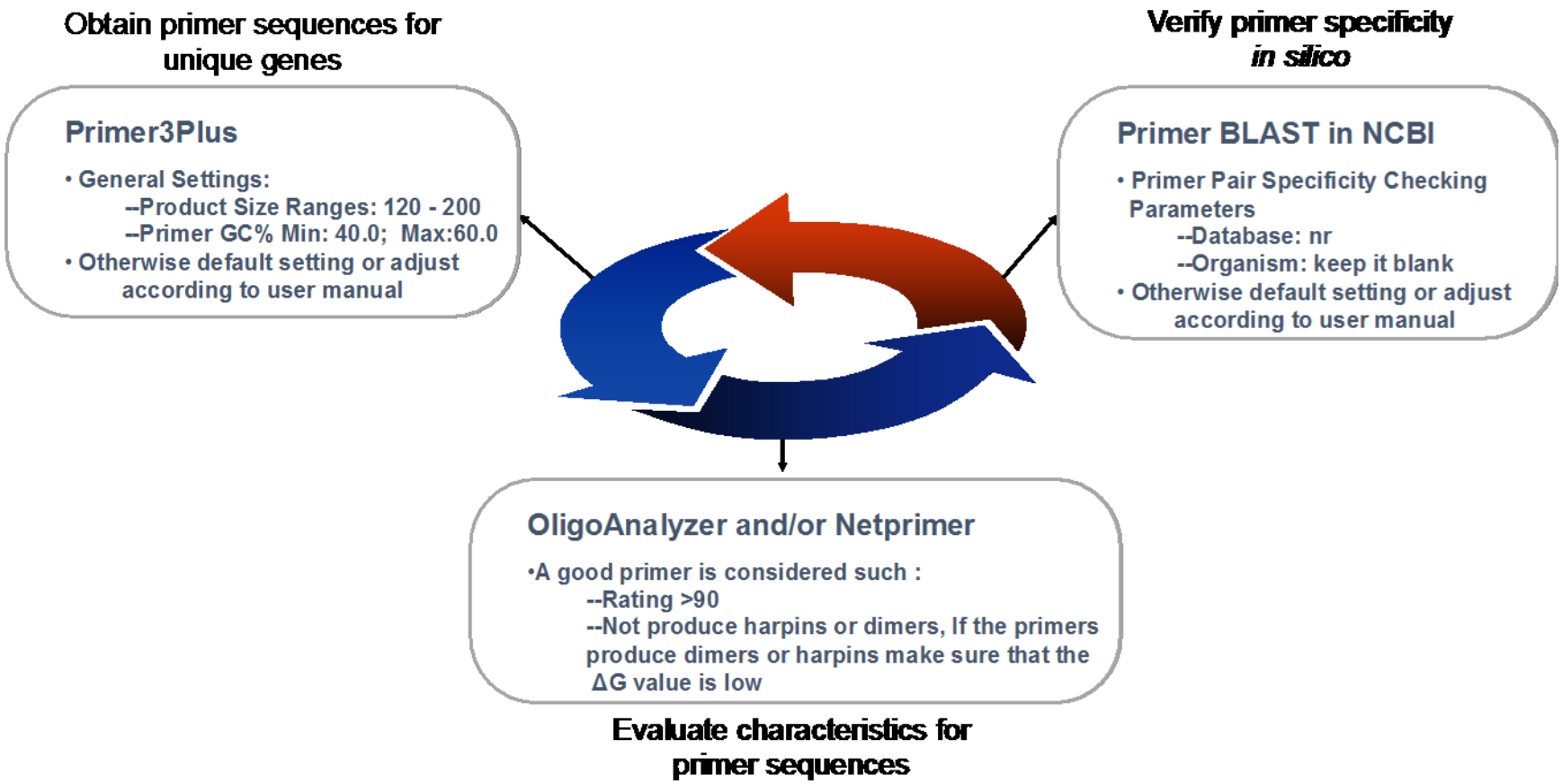


Figure 3. Primer design for *L. reuteri* PB-W1 and DSM 20016^{T} and validation *in silico.*

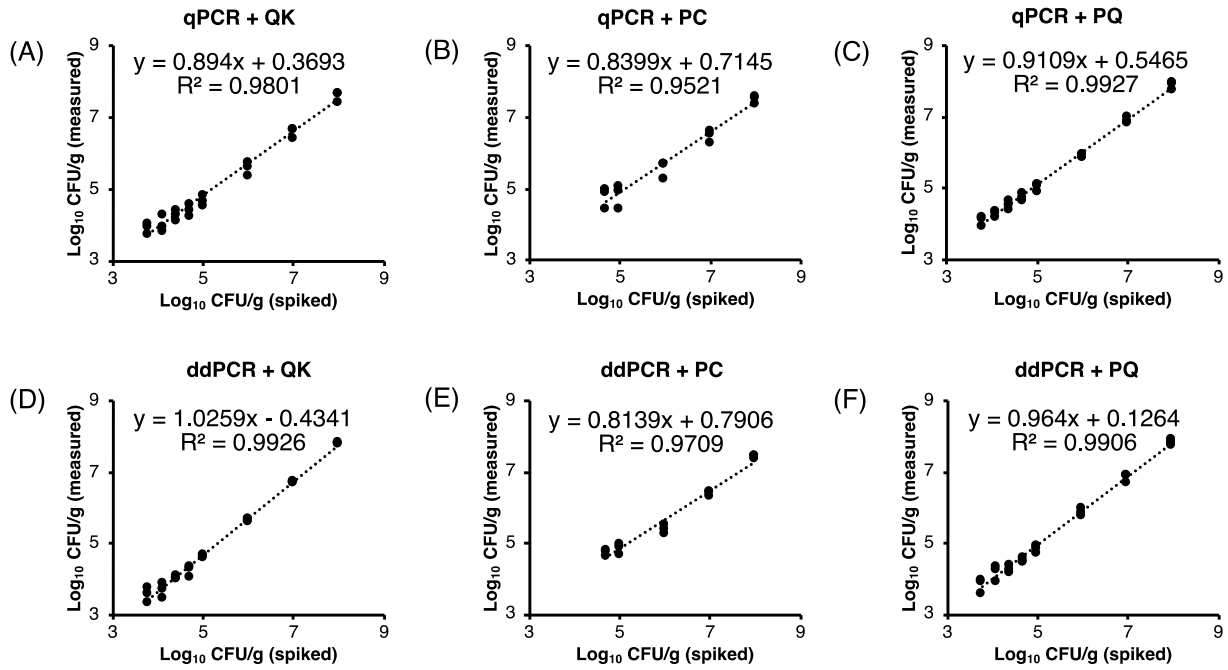
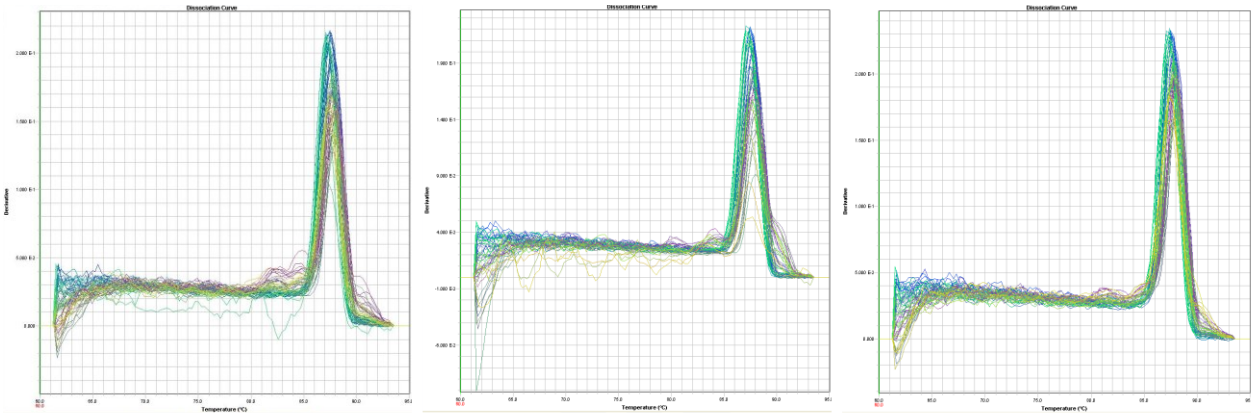


Figure 4. Linear regressions between measured CFU/g and spiked CFU/g for qPCR and ddPCR assays with different DNA extraction methods. (A-C), qPCR measurement combined with method QK, PC and PQ respectively. (D-F), ddPCR measurement combined with method QK, PC and PQ respectively. Spiked CFU/g fecal sample was estimated from quantitative culture on MRS plates. Each bacterial concentration was conducted in biological triplicates.



(A) (B) (C)

Figure 5. Melt curves for spiked stool samples in qPCR. (A-C), results obtained from spiked samples of three individuals and there was one specific peak at around 87.5℃ which indicated the amplification of target sequence for all reactions. Small bulges around the major peak in samples with low bacterial loads may imply the presence of background noise.

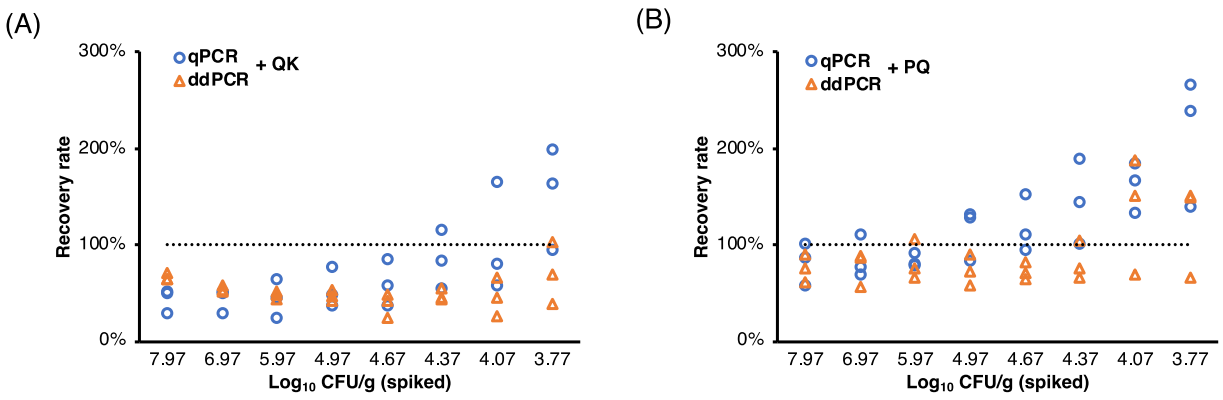


Figure 6. Comparing accuracy of qPCR and ddPCR. Recovery rate is the ratio between the cell numbers per gram feces measured and the cell numbers spiked (Wang *et al.*, 2018). (A) DNA extracted from spiked fecal samples using method QK in biological triplicates were analyzed by qPCR and ddPCR, respectively. (B) DNA extracted from spiked fecal samples using method PQ in biological triplicates were analyzed by qPCR and ddPCR, respectively. Results were compared with 100% recovery which is indicated by the dotted line.

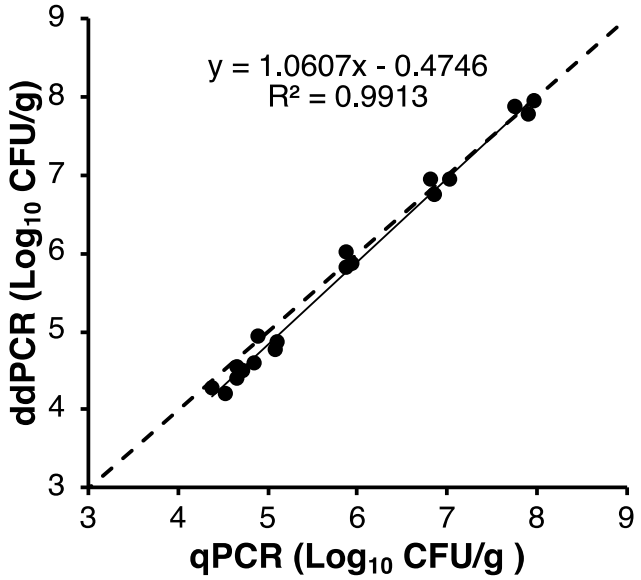


Figure 7. Correlation between qPCR and ddPCR in spiked samples using method PQ. Solid line, regression line; dash line, y = x. All dots are results obtained in qPCR and ddPCR.

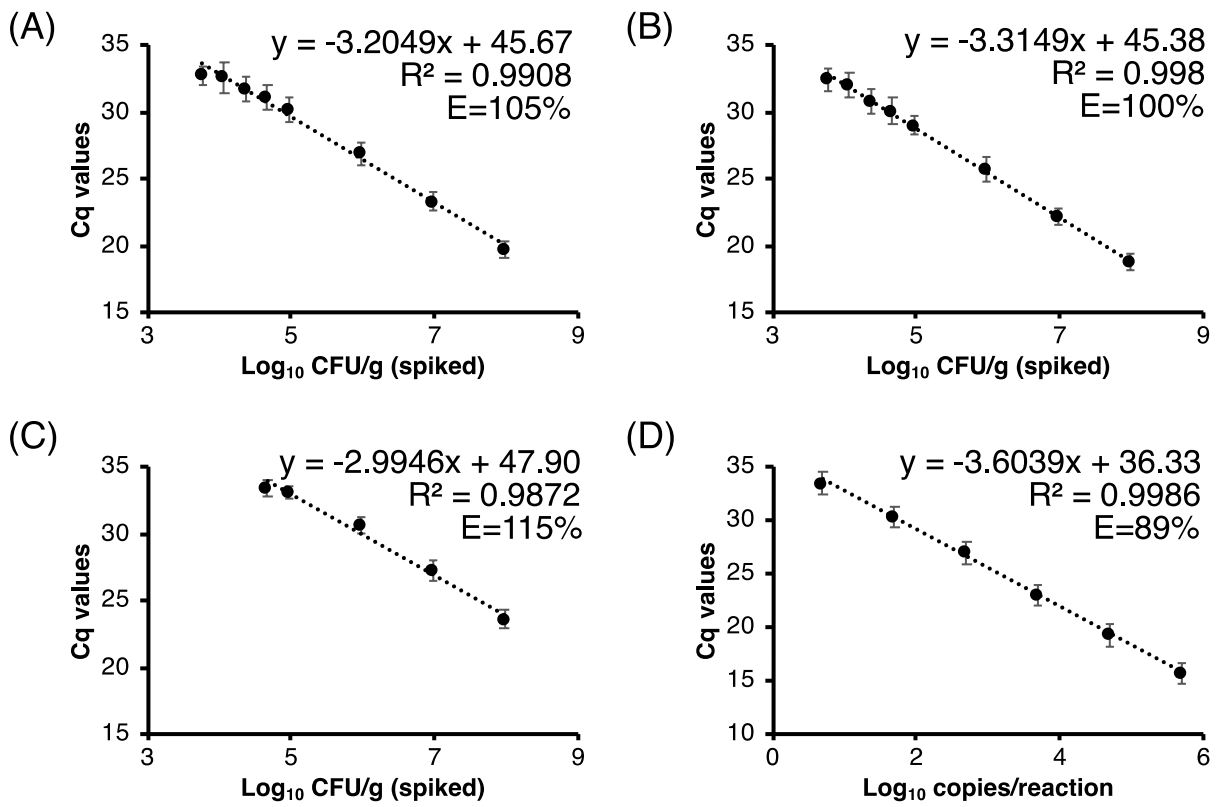


Figure 8. Standard curves constructed by plotting against the bacterial load spiked to each sample or the copy number estimated by spectrophotometer and corrected by ddPCR. Efficiency of PCR was calculated following the equation: $E = (10^{1/-slope} - 1) \times 100\%$. (A), DNA was extracted using method QK; (B) DNA was extracted using method PQ; (C), DNA was extracted using PC; each dot represents the average Cq values of three biological replicates measured in triplicates. (D), standards were established by serially diluting a certain amount of genomic DNA, and each dot represent the average Cq of six replicates from three independent runs.

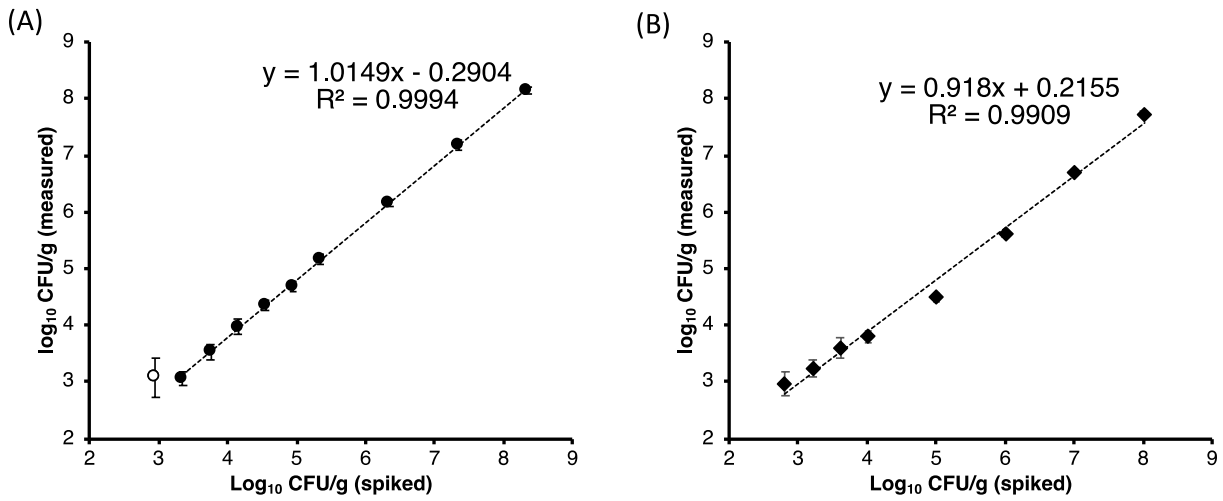


Figure 9. Correlation between the number of *L. reuteri* cells spiked to stool samples and the number measured using qPCR. (A), PB-W1 (in round); (B), DSM 20016^T (in diamond). The regression line was created between the spiking of 3.35 Log_{10} CFU/g and 8.34 Log_{10} CFU/g feces for PB-W1 and between 2.81 Log_{10} CFU/g and 8.01 Log_{10} CFU/g feces for DSM 20016^T, respectively, within which detection was reliable. As amplification products were still detectable but lacked reproducibility and linearity with an input of PB-W1 (2.96 Log_{10} CFU/g feces), it was shown in open round to highlight the difference. Each error bar displays the standard deviation from three replicates.

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