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DNA extraction leads to bias in bacterial quantification by qPCR

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Abstract

Quantitative PCR (qPCR) has become a widely used technique for bacterial quantification. The affordability, ease of experimental design, reproducibility, and robustness of qPCR experiments contribute to its success. The establishment of guidelines for minimum information for publication of qPCR experiments, now more than 10 years ago, aimed to mitigate the publication of contradictory data. Unfortunately, there are still a significant number of recent research articles that do not consider the main pitfalls of qPCR for quantification of biological samples, which undoubtedly leads to biased experimental conclusions. qPCR experiments have two main issues that need to be properly tackled: those related to the extraction and purification of genomic DNA and those related to the thermal amplification process. This mini-review provides an updated literature survey that critically analyzes the following key aspects of bacterial quantification by qPCR: (i) the normalization of qPCR results by using exogenous controls, (ii) the construction of adequate calibration curves, and (iii) the determination of qPCR reaction efficiency. It is primarily focused on original papers published last year, where qPCR was applied to quantify bacterial species in different types of biological samples, including multi-species biofilms, human fluids, and water and soil samples.

Keywords

Bacterial load quantification; gDNA extraction efficiency; qPCR; gDNA yield; Exogenous control; Calibration curve; qPCR reaction efficiency

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Author contribution NC, CAM, and CMT designed the mini-review structure. AL performed the literature review. AF, NC, and AL drafted the manuscript. AF prepared the figures. CAM and CMT critically reviewed the draft. All authors read and approved the manuscript.

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Introduction

Quantitative PCR (qPCR) has become a widely used technique for gene expression assessment, as well as for bacterial quantification (Kralik and Ricchi, 2017). The affordability, ease of experimental design, reproducibility, and robustness of qPCR experiments (Smith and Osborn, 2009) contribute to its success. In fact, qPCR is used in many other research fields beyond bacteriology and is even considered the gold standard in many applications (Pfaffl, 2010). While qPCR can be used to quantify DNA, RNA and even proteins, in this mini-review, the focus will be given to bacterial quantification in biological samples through genomic DNA (gDNA) amplification and quantification. Despite its success and technological potential, qPCR is not without its caveats, and the MIQE guidelines (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) were pivotal to improve reproducibility, data analysis, interpretation, and overall transparency of qPCR experimental reports (Bustin et al., 2009). As we will show in this mini-review, there are still a significant number of research articles that do not consider the main pitfalls of qPCR for bacterial quantification, which undoubtedly leads to biased conclusions (Bustin, 2010). The main objective of this mini-review is to perform an updated survey of recent literature to critically analyze how researchers conduct and report qPCR experiments for the quantification of bacteria in biological samples.

The general concept of qPCR beyond bacterial load quantification

When taking into consideration all required controls, the high reproducibility of a qPCR run, associated with a very low limit of detection, allows the accurate quantification of bacterial gDNA present in the initial biological sample (O'Connell et al., 2017a). As depicted in Fig. 1A, by performing a calibration curve relating initial bacterial concentration (before gDNA extraction) with the detection cycle threshold of a specific gene marker (present in the processed gDNA sample), qPCR data interpretation allows accurate determination of the initial bacterial load. However, the operative sentence here is "all the required controls."

As detailed in the MIQE guidelines (Bustin et al., 2009), qPCR experiments have two main issues that need to be properly addressed: those related to the extraction and purification of gDNA (McKee et al., 2015) and those related to the thermal amplification process (Ruiz-Villalba et al., 2017). While a lot of attention has been given to the latter, gDNA extraction troubleshooting is often oversimplified, mainly focusing on the gDNA yield and quality. However, it has been demonstrated by many studies that gDNA extraction efficiencies can vary significantly between experiments (Greathouse et al., 2019; Marotz et al., 2017; O'Connell et al., 2017b; Rezzonico et al., 2003). This can lead to biased quantification, as we have recently shown (Cerca et al., 2022). In addition to the inherent biological variability that occurs when growing in vitro cultures or collecting in vivo specimens, sample processing for qPCR experiments involves several steps, all of which can significantly contribute to variability (Caporaso et al., 2012; Greathouse et al., 2019; Sousa et al., 2014). It has been pointed out that the nucleic acid extraction step is the most important source of post-processing variability (Costea et al., 2017) as variation in the efficiency of this process will be further amplified during subsequent qPCR steps.

On one hand, if using in vitro technical replicates, with known bacterial concentrations, the variability in the gDNA extraction process can easily be observed by the differences in the total yield of gDNA obtained, as depicted in Fig. 1B. For example, after repeated extractions, if the user can anticipate a 1×10^8 CFU/mL bacterial pure culture yielding ~ 100 ng/μL of total DNA, then the user might exclude for downstream processing one extraction where only 5 ng/μL of total DNA was obtained and attribute this variation to some experimental flaw. However, if gDNA is extracted from an uncharacterized sample, which is often the goal of a qPCR experiment, DNA yield alone will not allow correct assessment of the initial bacterial load. Without the introduction of key experimental controls, it will not be possible to differentiate between a lower gDNA extraction efficiency and a lower initial bacterial load, since the same sample can yield two different gDNA concentrations, as depicted in Fig. 1C. This is even more relevant if human DNA is added to the mixture (Greathouse et al., 2019), as total DNA quantification will not allow inference of bacterial gDNA extraction yield. To tackle this issue, the addition of an exogenous DNA control before DNA extraction procedures, at a known concentration, has been identified as the best practice to normalize the inherent variations associated with gDNA extraction between samples (O'Connell et al., 2017a).

Impact of not assessing gDNA loss in bacterial quantification

Using a mock in vitro triple-species bacterial consortium, we recently demonstrated that the impact of not quantifying gDNA extraction efficiency in each reaction can significantly impact bacterial quantification (Cerca et al., 2022). As shown by others (Barton et al., 2006; Davis et al., 2019; Greathouse et al., 2019), at lower bacterial concentrations, the quantification error was significantly higher. Without considering gDNA extraction efficiency, we could find up to 46-fold under-representation of a particular species in the triple-species consortia. At higher bacterial concentrations, the quantification error was significantly lower, with no more than sixfold under-representation observed (Cerca et al., 2022). However, when we factored in the efficiency of gDNA extraction of each sample, as measured by the recovery rate of the exogenous control added before extraction, we were able to accurately (with less than 10% error) quantify each triple-species consortium with ~ 3×10^8 CFU/mL total bacterial load. When testing a consortium with only ~ 3×10^6 CFU/mL of total bacterial load, assessing gDNA extraction efficiency allowed no more than twofold under- or threefold over-representation of each species, which was significantly more accurate than the calculations excluding gDNA losses. Interestingly, different bacterial species incurred distinct gDNA losses.

Why determining qPCR reaction efficiency is not enough for accurate bacterial quantification?

One of the most basic rules of any qPCR experiment is the need to determine the qPCR reaction efficiency (Bustin et al., 2009). This is achieved by performing serial dilutions of a specific DNA sample (Svec et al., 2015). However, this is often mistakenly considered as a calibration curve for bacterial concentration determination. It is incorrectly assumed that the same linear response observed in the qPCR reaction efficiency mimics a linear calibration

curve for total bacterial load and qPCR cycle threshold of a specific bacterial species. As depicted in Fig. 1A, the relationship between a known DNA concentration and qPCR cycle threshold is only able to accurately assess the initial concentration of the DNA present in the processed sample, but this is hardly the same as assessing the initial bacterial concentration of the unprocessed sample (before gDNA extraction) to be analyzed. So, while determining qPCR reaction efficiency is mandatory for accurate bacterial quantification, as described in the MIQE guidelines, this does not replace the need to perform a proper bacterial gDNA extraction calibration curve.

In this regard, Longin and co-workers pointed out important recommendations to perform accurate bacteria quantification using qPCR (Longin et al., 2016). One of the recommendations included (i) the use of a bacteria as a spike in control added to the unprocessed sample before starting the DNA extraction. There are already a few studies that have successfully used this strategy as exogenous controls (Scarsella et al., 2021; Stoeckel et al., 2009). In these studies, bacteria were incorporated into the culture right before the centrifugation step to account for biomass losses during centrifugation. It is important to highlight that the selected exogenous bacterial species should be absent from the target samples under study. In most cases, this is possible to predict. However, if not, an alternative strategy could be the utilization of a genetically manipulated strain containing a synthetic sequence. Furthermore, attention should be given to differences in cell wall composition, since Gram-positive and Gram-negative bacteria can have different lysis efficiency (Ketchum et al., 2018; Mahalanabis et al., 2009; Wang et al., 2020), mainly due to a thicker peptidoglycan layer in Gram-positive bacteria (Auer and Weibel, 2017). Furthermore, when studying complex samples that might include both Gram-positive and Gram-negative bacteria, a mixture of Gram-positive and Gram-negative exogenous bacterial controls should be used, as recently proposed (Scarsella et al., 2021).

Other important recommendations indicated by Login et al. was (ii) the use of calibration curves constructed with gDNA isolated form pure cultures prepared at different bacterial concentrations, as gDNA extraction efficiency depends on initial bacterial concentration. In addition, (iii) dilution of pure cultures to construct calibration curves should include the biological matrix of the samples.

Is bacterial quantification by qPCR being properly performed and reported? A recent literature review

To perform this review, we focused on the analysis of the Materials and Methods sections of original papers published last year, from a wide range of research fields, by performing a PUBMED search with the key words “bacterial qPCR quantification,” “qPCR exogenous control,” and “bacterial qPCR normalization”. We found 95 research papers (Table 1). We centered our analyses on (i) the normalization of qPCR results by using exogenous controls, (ii) the construction of adequate calibration curves, and (iii) the determination of reaction efficiency, which are key steps for both DNA extraction and thermal amplification normalization steps. Surprisingly, (82%) of the surveyed manuscripts did not include a normalization strategy to account for bacterial loss and to tackle gDNA extraction

variability (Fig. 2). Among the studies where controls were included (8%), only 1% used proper exogenous controls, and the remaining 9% performed a normalization of gDNA concentration among samples. Regarding reaction efficiency, 53% of the studies did not consider this critical aspect, 25% did not determine the efficiency correctly, and thus, only 22% of the reports determined reaction efficiency properly. In the last aspect that we analyzed, the construction of calibration curves, we verified that only 4% of the studies performed proper calibration curves. In 45% of the studies analyzed, the calibration curves were constructed using dilutions of gDNA obtained from a single sample, a strategy routinely used to determine primer efficiency, instead of using gDNA obtained from samples with different bacteria concentrations, as discussed above. A large number (37%) of studies constructed calibration curves by cloning a gene into a plasmid, in order to determine absolute copy numbers. However, again, the calibration curve was constructed by performing dilutions of the same DNA sample. The most common errors found for each critical aspect of the qPCR are described in Fig. 3. It is important to highlight that none of the studies analyzed performed well in the three critical aspects evaluated. In this sense, we constructed a flowchart, Fig. 4, that summarizes the critical steps for absolute bacterial quantification by qPCR.

Concluding remarks

Our review of recent literature clearly shows that the use of qPCR for bacteria quantification is not properly described, with a lack of clarity to what concerns the description on how quantification was performed. Although the publication of MIQE guidelines were pivotal to improve scientific literature on the use of qPCR for a multitude of applications, they have focused on RNA extraction experiments and fail to mention the need to include the utilization of an exogenous control to determine gDNA extraction efficiency. However, this is of utmost importance to guarantee proper bacterial quantification by gDNA amplification. The inadequate use of qPCR for bacterial quantification will bias the results obtained, leading to the publication of inconsistent data and, consequently, misleading and erroneous conclusions. As such, it is vital to alert the scientific community of the pitfalls associated with the quantification of bacteria by qPCR and to establish guidelines on how to proceed to properly address the limitations of these assays and, this way, obtain reproducible, reliable, and meaningful data.

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Key points

- qPCR is a widely used technique used for absolute bacterial quantification.
- Recently published papers lack proper qPCR methodologies.
- Not including proper qPCR controls significantly affect experimental conclusions.

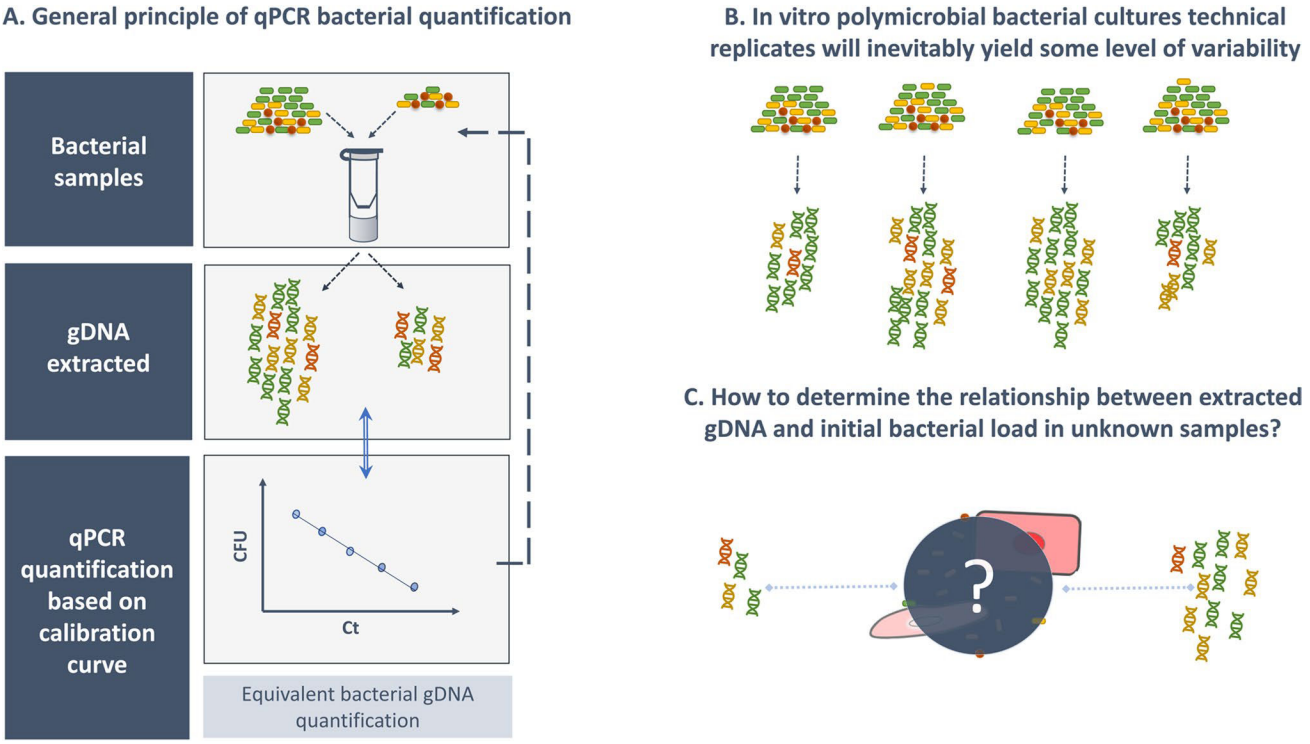

Fig. 1.

General concept and pitfalls behind bacterial load quantification by qPCR. Bacterial quantification by qPCR requires the preparation of a calibration curve, which includes gDNA isolated from samples with different concentrations of the bacterial species under study (**A**). However, due to the variable efficiency of the gDNA extraction procedure, each extraction, even from technical replicates, has inherent variability that can lead to biased bacterial load quantification (**B**). When the starting bacterial amount is known, gDNA loss, due to technical issues, can be easily detected. However, when the initial quantity of bacteria is unknown, it cannot be differentiated whether the variation detected was introduced by technical issues or if it was due to the initial bacterial load (**C**). As such, the addition of an exogenous control is imperative

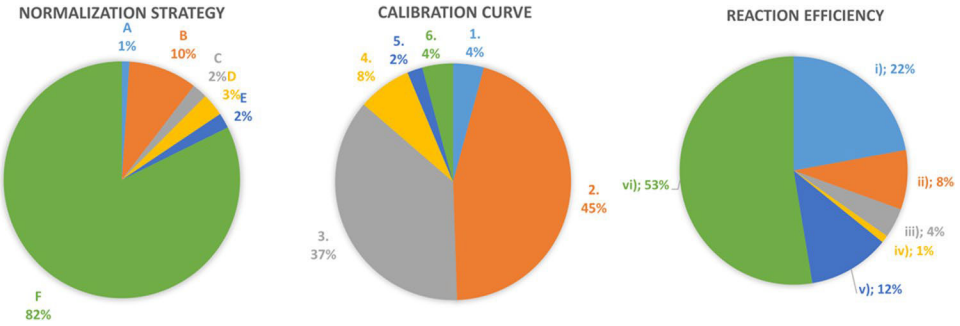

Fig. 2.

Graphical representation of the percentage (%) of studies presented in Table 1 that considered the three critical aspects for bacterial quantification by qPCR. The caption is the same as described in the Table 1 footer section

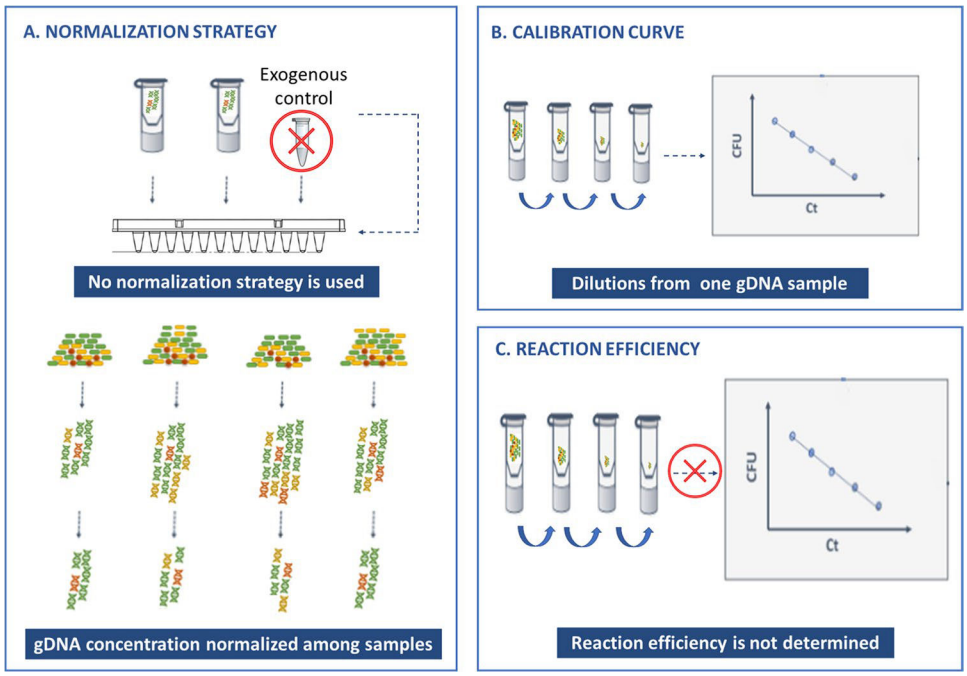

Fig. 3.

Common flaws when quantifying bacterial load using qPCR. In most of the studies surveyed, (**A**) DNA extraction normalization was not performed (or described) or was normalized by total gDNA concentration, which can bias the results. Often (**B**), the qPCR/bacterial load calibration curve is performed by diluting a known gDNA sample, but this fails to consider the different extraction efficiencies at different bacterial concentrations. Also (**C**) some studies failed to consider the qPCR reaction efficiency that needs to be determined for each primer set and varies according to reagent and equipment used

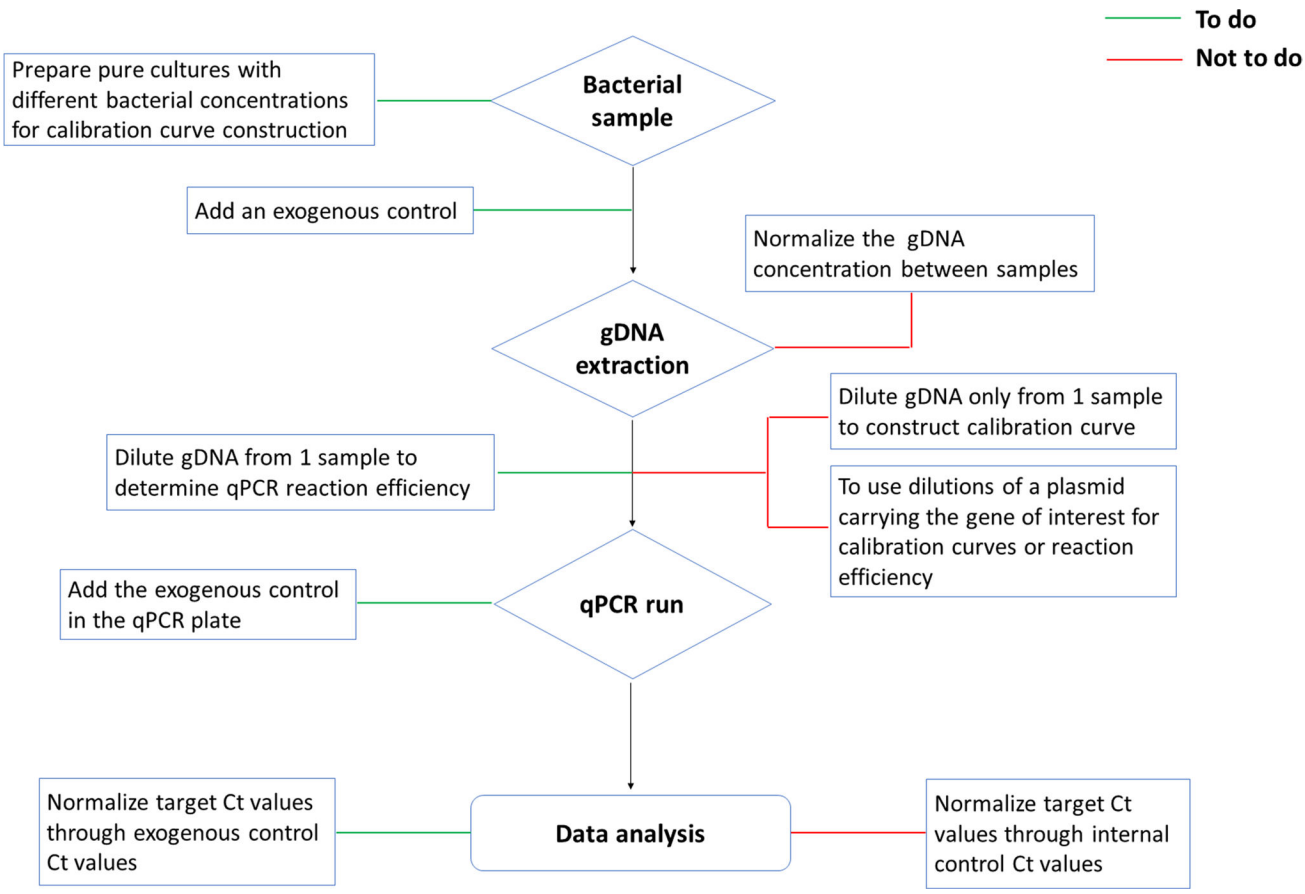

Fig. 4.

Flowchart highlighting the “do’s” and “don’ts” of procedures in bacterial quantification by qPCR. This scheme represents our recommended actions to perform a proper bacterial quantification by qPCR (green lines). It also includes common errors that are generally made (red lines) and should be avoided

Table 1

Literature survey of the three critical aspects for proper bacterial quantification by qPCR. The papers analyzed were all published in 2021

Scientific field	Purpose	Normalization strategy #	Calibration curve ###	Reaction efficiency ##	Reference
Vaginal infections	To quantify *G. vaginalis* before and after exposure to *Lactobacillus* spp.	F	2	vi)	(He et al., 2021a, b)
	To quantify *Candida* spp. and bacterial load in vaginal swabs	F	2	vi)	(McKloud et al., 2021)
	To quantify *Gardnerella* spp., *Lactobacillus* spp. and total bacterial population in vaginal swabs	E	2	i)	(Turner et al., 2021)
	To quantify the bacterial load and *G. vaginalis* in cervical fluid	D	3	vi)	(Kacerovsky et al., 2021)
	To quantify *G. vaginalis* and *Lactobacillus* spp. in mouse vaginal tissue	B	4	iv)	(Selis et al., 2021)
	To quantify *Enterobacteriaceae*, *Staphylococcus* spp., and *Streptococcus* spp. in vaginal swabs	F	2	vi)	(Oh et al., 2021)
Wound infections	To quantify different bacteria in a polymicrobial biofilm	D	2	vi)	(Li and Wu, 2021)
	To quantify *Staphylococcus aureus* in mice tissues	F	4	vi)	(Do Pham et al., 2021)
Gastrointestinal infections/ microbiota	To quantify *Clostridioides difficile* in a polymicrobial biofilm	B	3	vi)	(Normington et al., 2021)
	To quantify bacterial load and nine bacteria in feces samples	B	3	vi)	(Tonon et al., 2021)
	To quantify *Prevotella histicola* in human duodenal biopsies	F	2	i)	(Balakrishnan et al., 2021)
	To quantify bacterial load in cecum content and feces	F	3	vi)	(Taibi et al., 2021)
	To quantify enterotoxigenic *E. coli* F4 in colon and ileal mucosal samples of piglets	F	2	vi)	(Rodríguez-Sorrento et al., 2021)
	To quantify *Bacteroides vulgatus* in fecal samples	F	3	ii)	(Maier et al., 2021a, b)
	To quantify fungal load in stool samples	B	2	vi)	(Boutin et al., 2021)
	To quantify bacteria and fungi in honeybee gut samples	F	3	vi)	(Callegari et al., 2021)
Pulmonary infections	To quantify *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* in respiratory samples from cystic fibrosis patients	F	3	vi)	(Pittet et al., 2021)
	To quantify three bacteria associated with ventilator-associated pneumonia in dual-species biofilms with *Candida albicans*	F	2	i)	(Luo et al., 2021)
	To quantify bacteria in bronchoalveolar fluid samples	F	3	vi)	(Invernizzi et al., 2021)
Oral biofilms	To determine the composition of polymicrobial biofilms	F	3	vi)	(Redanz et al., 2021)
	To determine and quantify bacteria in dental caries	F	2	vi)	(Chen et al., 2021)
	To quantify bacteria in a polymicrobial biofilm	F	3	vi)	(Verspecht et al., 2021a)
	To detect and quantify bacteria in polymicrobial biofilms from saliva and dental plaque	B	4	vi)	(Oliveira et al., 2021)
	To quantify pathogenic bacteria in subgingival plaque and saliva samples	F	2	vi)	(Sereti et al., 2021)

Scientific field	Purpose	Normalization strategy #	Calibration curve ###	Reaction efficiency ##	Reference
	To quantify bacteria and fungi in a tooth model	F	4	i)	(Leelapompisid et al., 2021)
	To quantify bacteria in a dual-species biofilm	B	5	vi)	(Millones-Gómez et al., 2021)
	To quantify bacteria in a polymicrobial biofilm	F	3	vi)	(Verspecht et al., 2021b)
	To quantify bacteria in a multi-species community and bacteria present in ligatures placed around teeth of mice	C	2	vi)	(Hoare et al., 2021)
	To quantify bacteria in a polymicrobial biofilm	F	2	vi)	(Chathoth et al., 2021)
	To quantify *Porphyromonas gengivalis* in oral samples	F	2	vi)	(Franciotti et al., 2021)
	To quantify two bacteria in oral samples and colon tissue	F	2	vi)	(Pignatelli et al., 2021)
	To quantify microbial load in saliva samples	F	2	vi)	(Marotz et al., 2021)
	To quantify four bacteria in subgingival samples	F	2	v)	(Cuenca et al., 2021)
	To quantify bacterial load, and two different bacteria in periapical tissue	F	2	vi)	(Bordagaray et al., 2021)
	To quantify *Streptococcus mutans* and *Candida albicans* in oral samples	F	2	vi)	(Yang et al., 2022)
Soil microbiota	To quantify bacterial and fungal load and *Fusarium oxisporum* in soil samples	F	6	v)	(Zhu et al., 2021a, b)
	To quantify bacterial and fungal load	F	2	i)	(Ammitzboll et al., 2021)
	To quantify ammonia-oxidizing archaea and bacteria in soil samples	F	3	v)	(Dai et al., 2021)
	To quantify *Streptomyces bottropensis* and *Brevibacillus laterosporus* in soil samples	F	3	v)	(Li et al., 2021a, b, c)
	To quantify bacterial load in wheat root samples	F	3	v)	(Usyskin-Tonne et al., 2021)
	To quantify ammonia-oxidizing archaea and bacteria in soil samples	F	3	v)	(Samaddar et al., 2021)
	To quantify bacterial and fungal load in soybean soil samples	F	3	v)	(Gao et al., 2021a, b)
	To quantify *Calonectria ilicicola* in soybean soil samples	F	2	vi)	(Ochi and Kuroda, 2021)
	To quantify ammonia-oxidizing archaea and bacteria in soil samples	F	3	vi)	(He et al., 2021a, b)
	To quantify *F. oxysporum*, bacterial and fungal load, ammonia-oxidizing archaea and bacteria in soil samples	F	3	ii)	(Liu and Zhang, 2021)
	To quantify bacterial load, ammonia-oxidizing archaea and bacteria in soil samples	F	3	vi)	(Li et al., 2021a, b, c)
	To quantify bacterial load in soil samples	F	3	vi)	(Nogrado et al., 2021)
	To quantify bacterial load in soil samples	F	2	v)	(Han et al., 2021)
	To quantify bacterial load in dry soil samples	F	3	ii)	(Zhao et al., 2021)
	To quantify bacterial load in soil samples	F	3	ii)	(Wang et al., 2021a, b)
	To quantify bacterial load in soil samples	F	3	ii)	(Zhu et al., 2021a, b)
	To quantify bacterial load in soil samples	F	3	vi)	(Zhang et al., 2021a, b)

Scientific field	Purpose	Normalization strategy #	Calibration curve ###	Reaction efficiency ##	Reference
	To quantify *Brevibacillus laterosporus* and *S. bottropensis* in soil samples	F	3	v)	(Li et al., 2021a, b, c)
	To quantify ammonia-oxidizing archaea and bacteria in soil samples	F	3	vi)	(Wei et al., 2021)
	To quantify ammonia-oxidizing archaea, ammonia-oxidizing bacteria, *Nitrobacter* and *Nitrospira* spp. in soil samples	F	3	vi)	(Yin et al., 2021)
	To quantify *Rhizoctonia solani* and *Rhizoctonia solani* AG1-IB in lettuce and soil samples	D	2	i)	(Wallon et al., 2021)
	To quantify bacterial load in soil samples	B	2	vi)	(Alvarez et al., 2021)
	To quantify *F. oxysporum* f. sp. cubense, *Fusobacterium solani* and *Aspergillus fumigatus* in soil samples	F	3	vi)	(Yuan et al., 2021)
	To quantify bacterial and fungal load, *Ralstonia solanacearum* and *F. oxysporum* f. sp. *Lycopersici* in soil samples	F	6	vi)	(Deng et al., 2021)
	To quantify *Azospirillum brazilense* in soil samples	F	1	iii)	(Urrea-Valencia et al., 2021)
	To quantify *Pseudomonas protegens* and *Bacillus subtilis* in soil samples	F	3	ii)	(Zhang et al., 2021a, b)
Bacterial contamination on surfaces	To quantify *Lactobacillus delbrueckii* subsp. Bulgaricus in surface samples	F	2	i)	(Wang et al., 2021a, b)
	To quantify four *Staphylococcus* spp. in hospital surfaces	F	2	vi)	(Gismondi et al., 2021)
	To quantify *Candida auris* in skin swabs and hospital surfaces	F	4	vi)	(Sexton et al., 2021)
Food quality control / Foodborne pathogens	To quantify three *Lactobacillus* spp. in sourdough bread	F	2	i)	(Baek et al., 2021)
	To quantify *Lactobacillus* spp. in fermented dairy products samples	F	2	i)	(Yang et al., 2021)
	To quantify *Brucella* spp. in milk and cheese samples	F	2	vi)	(Marouf et al., 2021)
	To quantify seven proteolytic *Pseudomonas* spp. in raw milk samples	F	1	iii)	(Maier et al., 2021a, b)
	To quantify *Nucleospora cyclopteri* in fish and blood samples	F	2	i)	(Naung et al., 2021)
	To quantify different bacteria in meat samples	F	2	i)	(Bahlinger et al., 2021)
	To quantify *Colletotrichum acutatum* in olive fruit samples	F	3	v)	(Azevedo-Nogueira et al., 2021)
	To quantify *Lysteria monocytogenes* in meat samples	F	1	iii)	(Labrador et al., 2021)
	To quantify Shiga toxin-producing *Escherichia coli* in meat samples	F	2	vi)	(Rey et al., 2021)
	To quantify *Campylobacter coli* in meat samples	F	2	i)	(Lazou et al., 2021)
	To quantify *Brochothrix thermosphacta* in fish samples	F	2	i)	(Bouju-Albert et al., 2021)
	To quantify *Streptococcus iniae* in fish samples	F	2	i)	(Torres-Corral and Santos, 2021)
	To quantify *Cronobacter sakazakii* in spiked powdered infant formula samples	F	2	i)	(Gao et al., 2021a, b)
	To quantify four *Campylobacter* spp. in meat samples	F	2	i)	(Vizzini et al., 2021)
	To quantify *Salmonella* spp. in poultry floor dust	F	1	i)	(Ahaduzzaman et al., 2021)
	To quantify six bacteria genera and four bacteria phyla in milk samples	F	2	vi)	(Sanjulián et al., 2021)

Scientific field	Purpose	Normalization strategy #	Calibration curve ###	Reaction efficiency ##	Reference
Waterborne pathogens	To quantify bacteria in polymicrobial biofilms present in a drinking water distribution system	C	3	i)	(Fu et al., 2021)
	To quantify several enteric opportunistic pathogens in influent and recycled water	F	2	i)	(Drigo et al., 2021)
	To quantify *Enterococcus* spp. and *Salmonella* spp. in water, sand, and sediments samples	F	3	ii)	(Li et al., 2021a, b, c)
	To quantify *Helicobacter pylori* and *Legionella* spp. in filtered water samples	F	5	vi)	(Ribes et al., 2021)
	To quantify pathogenic fungi, enteric and opportunistic pathogens	F	3	ii)	(Hu et al., 2021)
	To quantify several bacteria in water samples	F	1	iii)	(Ambili and Sebastian, 2021)
Other fields	To detect and quantify bacteria in breast implant samples	E	2	vi)	(Crowe et al., 2021)
	To quantify bacteria in bovine digital dermatitis	B	3	v)	(Caddey et al., 2021)
	To quantify bacterial load in livestock fecal manure samples	F	3	vi)	(Wongsaroj et al., 2021)
	To quantify *Zobellia genus* in macroalgae surface	F	2	i)	(Brunet et al., 2021)
	To quantify *Campylobacter fetus* subsp. fetus and *Salmonella enterica* subsp. *enterica* serovar Typhimurium in bovine endometrial cells	F	4	vi)	(Muzquiz et al., 2021)
	To quantify five bacteria in cosmetic cream samples	F	2	i)	(Bermond et al., 2021)
	To quantify bacterial and fungal load in dust samples	F	4	vi)	(Haines et al., 2021)
	To quantify bacterial load in skin swabs, and skin samples from patients with atopic dermatitis	F	2	vi)	(Edslev et al., 2021)
	To quantify bacterial load in milk, feces and blood samples from healthy cows and cows suffering from bovine mastitis	A & B	6	vi)	(Scarsella et al., 2021)

#Normalization strategy—A—An exogenous control was used; B- Initial gDNA concentration normalization among samples; C—gene copy numbers were normalized by the filtrate membrane surface area / ligature length; D-16 s rRNA gene (internal control) was used; E—an exogenous control was used only in the qPCR run. F- No normalization strategy was used

##Calibration curve—1—Calibration curve performed using gDNA isolated from pure cultures with different initial concentrations, or pure cultures mixed with sample background of interest; 2—calibration curve performed with gDNA dilutions from one sample; 3—calibration curve performed by cloning a gene into a plasmid to determine absolute copy numbers by performing dilutions of the sample; 4-it is not clear whether the calibration curve was performed using dilutions of one extraction or different extractions from different bacterial concentrations; 5—standard curve was mentioned but it was not explained how it was constructed. 6 -No standard curve was mentioned

###Reaction efficiency—i—gDNA from one sample; ii—dilution of one sample with a plasmid containing the gene of interest; iii—gDNA extracted from samples with different concentrations of bacteria; iv—it is not clear whether qPCR primer efficiency was determined by using dilutions of one gDNA sample or from gDNA samples isolated from samples with different initial bacteria concentration; v—considered but the procedure to determine the efficiency was not mentioned; vi—not mentioned