



Variability of RNA quality extracted from biofilms of foodborne pathogens using different kits impacts mRNA quantification by qPCR. *Current Microbiology* (2012) 65:54–9

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Abstract

The biofilm formation by foodborne pathogens is known to increase the problem related with surface disinfection procedure in the food processing environment and consequent transmission of these pathogens into the population. Messenger RNA has been increasingly used to understand the action and the consequences of disinfectants in the virulence on such biofilms. RNA quality is an important requirement for any RNA-based analysis since the quality can impair the mRNA quantification. Therefore, we evaluated five different RNA extraction kits using biofilms of the foodborne pathogens *L. monocytogenes*, *E. coli* and *S. enterica*. The five kits yielded RNA with different quantity and quality. While for *E. coli* the variability of RNA quality did not affect the quantification of mRNA, the same was not true for *L. monocytogenes* or *S. enterica*. Therefore, our results indicate that not all kits are suitable for RNA extraction from bacterial biofilms and thus, the selection of RNA extraction kit is crucial to obtain accurate and meaningful mRNA quantification.

Introduction

Foodborne pathogens are responsible for, approximately, 9.5 million illnesses, 55.961 hospitalizations and 1.351 deaths in the United States [21]. Within the known foodborne pathogens, *Escherichia coli*, *Salmonella enterica* and *Listeria monocytogenes* are among the most common [21]. Surface contamination in food processing environments by these bacteria, and consequent inadequate or ineffective disinfection procedure is one of the direct causes of food spoilage and foodborne pathogens transmission into the population [8;11;16]. Biofilms, defined as tri-dimensional communities of bacteria surrounded by extra-polymeric substances such as polysaccharides, proteins, lipids and DNA, are regarded as an important virulence factor in food processing environment. Previous studies demonstrated that biofilms are less susceptible to sterilization procedures such sanitizers, than their planktonic counterparts [3;5;9]. Therefore, biofilm formation by foodborne pathogens increases the inefficiency of sterilization treatment [4;13] and thus, the risk of pathogens transmission into the population.

Studying gene expression was shown to be an important analysis tool to be used since it allows to evaluate how new sanitizers impact the virulence of foodborne bacterial strains [17]. In order to assess specific changes in the bacterial physiology, the correct quantification of specific messenger RNA (mRNA) from bacterial biofilms is an important requirement. Currently, there are plenty of different RNA extraction kits available and it has been shown that distinct kits can yield RNA with different levels of quality. This can be due to inherent characteristics of the kit and/or due to the nature of the sample [7;14;19]. Biofilms are communities of bacteria embedded on extracellular matrix, which is estimated to comprise up to 90% of the total biofilm biomass [6]. Polysaccharides, one of the major components of many bacterial biofilm matrices, seems to difficult the bacterial cell lysis and the nucleic acids, once

purified, may still contain inhibitory substances that will influence the accuracy and reproducibility of mRNA quantification [10;20]. Therefore, the aim of this work was to compare the performance of five commercially available RNA extraction kits, namely: FastRNA® Pro Blue (MP Biomedicals, Irvine, CA, US), Illustra RNAspin Mini (GE Healthcare, Upsala, Sweden), PureZOL™ RNA isolation reagent (Bio-Rad, Hercules, CA, US), PureLink™ RNA Mini Kit (Invitrogen, San Diego, CA, US) and GenJET™ (Fermentas, Ontario, Canada) using samples from the biofilm-forming foodborne pathogens *L. monocytogenes*, *E. coli* and *S. enterica*.

Material and Methods

Bacteria and biofilm formation conditions

In this work, 3 different foodborne biofilm-forming bacteria were used: *Listeria monocytogenes* CECT 4031T, *Escherichia coli* K12 substrain MG 1655 and *Salmonella enterica* serovar enteritidis NCTC 13349. Bacterial biofilms were grown as previously described [2;17]. Briefly, one single colony of *L. monocytogenes* was inoculated in 2 mL Tryptic Soy Broth (TSB) (Oxoid, Cambridge, UK) and *E. coli* and *S. enterica* in Luria-Bertrani (LB) Broth (Merck, NJ, US), from Tryptic Soy Agar plates not older than 2 days and grown at 37°C in a shaker rotator at 120 rpm for 24 (±2) hours. Then, 1:200 dilution was performed in fresh TSB and incubated in a 24-well plate (Orange Scientific, Braine-L'Alleud, Belgium) at 37°C, 100 rpm for 24 (±2) hours. Biofilms were washed with 0.9% NaCl to remove planktonic cells before RNA extraction. Biofilm biomass was quantified by optical density (OD) at 595 nm by the crystal violet staining method as described before [2]. This experiment was performed in triplicates.

RNA extraction and quality

Total RNA was isolated according to the manufacturer's instructions, with the following optimization: when appropriated, enzymatic lysis was performed during 60 min at 37°C with 15mg/mL of lysozyme. The RNA extraction kits were selected based on their different extraction principles: organic extraction with mechanical and chemical lysis (FastRNA® ProBlue (MPBiomedicals), organic extraction with enzymatic lysis (PureZOL™ RNA isolation reagent (Bio-Rad)) and silica membrane extraction with enzymatic lysis (Illustra RNAspin Mini (GE Healthcare), PureLink™ RNA Mini Kit (Invitrogen) and GenJET™ (Fermentas)). The final RNA fraction was obtained by suspending or eluting in 45 µL of RNase free water. To digest possible contaminating genomic DNA, DNase I (Fermentas) treatment was performed following the manufacturer's instructions. Briefly, 5 µL (10x) of reaction buffer and 2 µL DNase I were added to the extracted RNA and incubated at 37°C for 30 minutes. After that, 5 µL of 25mM EDTA was added and incubated at 65°C for 10 minutes, in order to inactivate the DNase I enzyme. RNA yield (ng/µL) and purity (A_{260}/A_{280} and A_{260}/A_{230}) were determined using a NanoDrop 1000™ (Thermo Scientific, MA, US). RNA integrity was verified by loading the samples in a 1% agarose gel run at 80 V for 60 min and stained with ethidium bromide. Gels were visualized using a GelDoc2000 (Bio-Rad). RNA samples were stored at -80°C for further analysis. Each RNA extraction was performed two to four times.

Quantitative Real Time-PCR (qPCR)

For the quantification of gene expression by qPCR, total RNA was reverse transcribed to complementary DNA (cDNA) using iScript™ cDNA Synthesis Kit (Bio-Rad) following the manufacturer's instructions. Briefly, 7.5 µL of total RNA was mixed with 2 µL iScript reaction buffer (5x) and 0.5 µL of reverse transcriptase. The samples were incubated at 25°C for 5 minutes, 42°C for 30 minutes and 85°C for 5 minutes. Oligonucleotide primers for the amplification of 16S rRNA, a housekeeping gene, and specific virulence genes of each bacterium tested (Table 1) were designed using Primer3 software [18] using as templates the genomes with the following accession numbers CP002816.1, NC_013353.1 and AM933172.1. Primers efficiency was determined by the dilution method and using a temperature gradient. At the selected annealing temperatures the primer-pairs had equivalent priming efficiencies. Two µL of each primer pair, at 10 µM, were added to 10 µL of (2x) SsoFast™ EvaGreen supermix (Bio-Rad), 6 µL of RNase free water and 2 µL of 1:20 cDNA dilution. The experiment was performed in

CFX96™ real time PCR system (Bio-Rad) using the following cycling parameters: 30 seconds at 94°C followed by 40 repeats of 5 seconds at 94°C, 10 seconds at 53°C (for *L. monocytogenes* primers) or 50°C (for *S. enterica* primers) or 60°C (for *E. coli* primers) and finally 15 seconds at 72°C. Neither unspecific product nor primer dimer formation was observed in the melting curves. The absence of genomic DNA contamination was assessed by including a control where the reverse transcriptase reaction did not occur. The cycle threshold (Ct) detection of each gene was determined using the standards parameters of the software. The gene expression quantification was plotted using the Ct values obtained by each RNA extraction kits/ bacteria combination. The linearity (r^2) of gene expression quantification was then determined assuming a linear regression. The qPCR was performed two times with triplicates.

Statistical analysis

All the assays were compared using one-way analysis of variance (ANOVA) with Tukey multiple comparisons test and also the unpaired sample t-test, using SPSS software (Statistical Package for the Social Sciences). Student's t-test was applied to all experimental data for rejection of some experimental values. All tests were performed with a confidence level of 95%.

Table 1 | Sequences of the oligonucleotide primers used for qPCR amplification assay. The theoretical melting temperature (T_m), the amplicon size and the priming position within the gene are indicated.

Bacterial species	Gene	Primer sequence (5'-3')	
		Forward	Reverse
<i>L. monocytogenes</i>	16S	GGAGCATGTGGTTTAATTCG	CCAACAAATGCTGGCAACT
	<i>prfA</i>	GGTAGCCTGTTCGCTAATGA	TAACCAATGGGATCCACAAG
<i>E. coli</i>	16S	CGGACGGGTGAGTAATGTCT	TCAGACCAGCTAGGGATCGT
	<i>tyrB</i>	CGTCAGGAATTGGTGAAGGT	TGGCGATGAGATAGACACCA
<i>S. enterica</i>	16S	CAGAAGAAGCACCGGCTAAC	GACTCAAGCCTGCCAGTTTC
	<i>ropS</i>	GAATCTGACGAACACGCTCA	CCACGCAAGATGACGATATG

Results and Discussion

Biofilm formation quantification

The purpose of this study was to compare the performance of different RNA extraction methods using bacterial biofilms. Considering that initial amount of bacterial cells used for RNA extraction will impact, at some extent, the total RNA quantity, it is important to quantify the biofilms used for RNA extraction. However, the relationship between amount of sample and RNA yield is not completely linear and each kit normally has an optimal range of sample concentration. In a comparison study using several different RNA extraction kits in rabbit blood samples it was found that in one of the extraction kits used, too much concentrated sample would result in lower RNA yields. Still, the majority of the cases reported indicated that a lower initial sample concentration would result in lower yields of RNA [14]. The amount of biofilm formed by each strain is presented in Fig 1. *L. monocytogenes* was clearly the strain with a thicker biofilm (OD595nm \approx 2) statistically significant $p < 0.05$) while *E. coli* and *S. enterica* formed thinner biofilms

(OD_{595nm} ≈0.5), that were easily detached from the surface. Due to the intrinsic differences in bacterial species biofilm formation ability and matrix composition we do not compared the performance of the RNA extraction kits between the different organisms.

As our experiments aimed to study the outcome of RNA quality in gene expression quantification inside bacterial biofilm communities, and since most biofilms cells show distinct mRNA expression profile depending on the region of the biofilm [23], we did not try to further explore a possible optimization of the process by using lower cell concentration, as this could potentially result in high variability from one biological replicate to another.

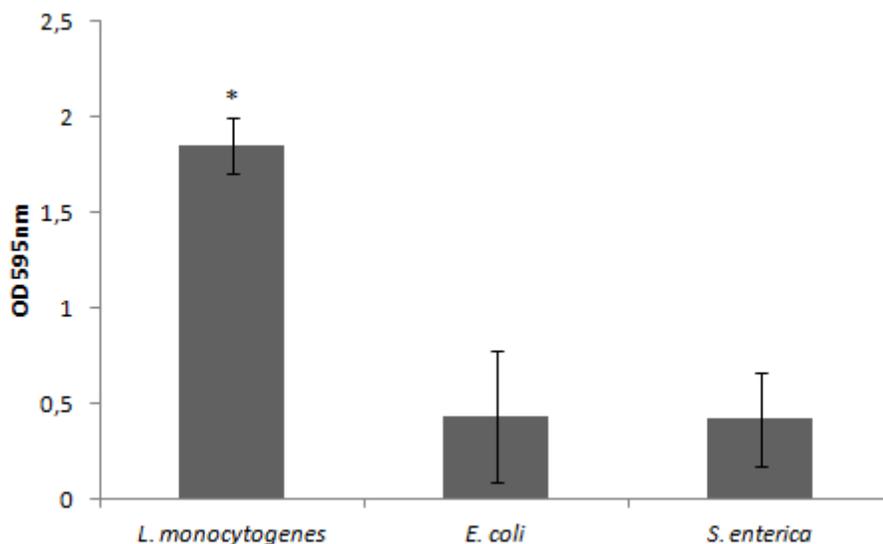


Figure 1 | Biofilm quantification by crystal violet staining method. The bars and the points represent the mean plus or minus standard deviation of three independent experiments with eight different biofilms per experiment (* statistically significant $P < 0.05$).

RNA yield, purity and integrity

When determining the RNA quality, a few parameters such as RNA yield, purity and integrity are normally considered [1]. Despite the inherent factors related with each specific RNA extraction kits, the samples can present intrinsic properties that can lead to the extraction of different quantity and quality of RNA. Although for many applications the minimum required quantity of RNA can be relatively low, when the aim of the experiment is to detect low expressing genes, a reduced yield of extraction could place these genes below the limit of detection. Thus, RNA quantity is one important requirement to consider when choosing a proper RNA extraction kit. A known issue regarding RNA yield is related with the increased difficulty in lysing Gram-positive bacteria [7]. According to the manufacturer instructions, aiming to optimize the RNA extraction of Gram-positive bacteria, we increased the concentration of lysozyme to 15 mg/mL as well as the lysis step for 60 minutes. This optimization increased the yield of total RNA from 2 to 4 fold (data not shown). Interestingly, for each of the bacterial species addressed in this study, a different kit resulted in higher RNA quantity: PureLink™ was the best kit for *E. coli* (240 ng/μl), Illustra RNAspin for *S. enterica* (140 ng/μl) and FastRNA® for *L. monocytogenes* (274 ng/μl) (Fig. 2).

Besides the importance of the quantity, the sample purity is another important requirement in RNA extraction procedures, since the presence of inhibitory compounds can influence the accuracy of the downstream applications [12]. The RNA purity is usually evaluated by determining the absorbance ratios A_{260}/A_{280} and A_{260}/A_{230} [18]. Pure RNA is expected to have the referred ratios above 1.8. An A_{260}/A_{280} lower than 1.8 indicates the presence of protein contamination while, an A_{260}/A_{230} lower than 1.8 indicates

the presence of polysaccharides from the biofilm matrix, and/or other inhibitory compounds such as phenol and chaotropic salts, possibly introduced during RNA extraction procedure [24]. When working with complex microbial communities such as biofilm, RNA purity is often impaired mainly due to the presence of the biofilm matrix components, such as proteins and polysaccharides [7]. In general, all the RNA extraction kits/bacteria combinations presented an A_{260}/A_{280} ratio between the range accepted for pure RNA. The only exception was the kit from GenJET™ in *L. monocytogenes* where the values were under 1.8 (Fig. 2). On the other hand, regarding the A_{260}/A_{230} ratio results, we observed that most of the kits used, especially PureZOL™, were ineffective removing the polysaccharides from the biofilm samples and/or other inhibitory compounds during RNA extraction procedure. The only exceptions were PureLink™ in *E. coli* and GenJET™ in *S. enterica* (Fig. 2) that showed A_{260}/A_{230} above 1.8.

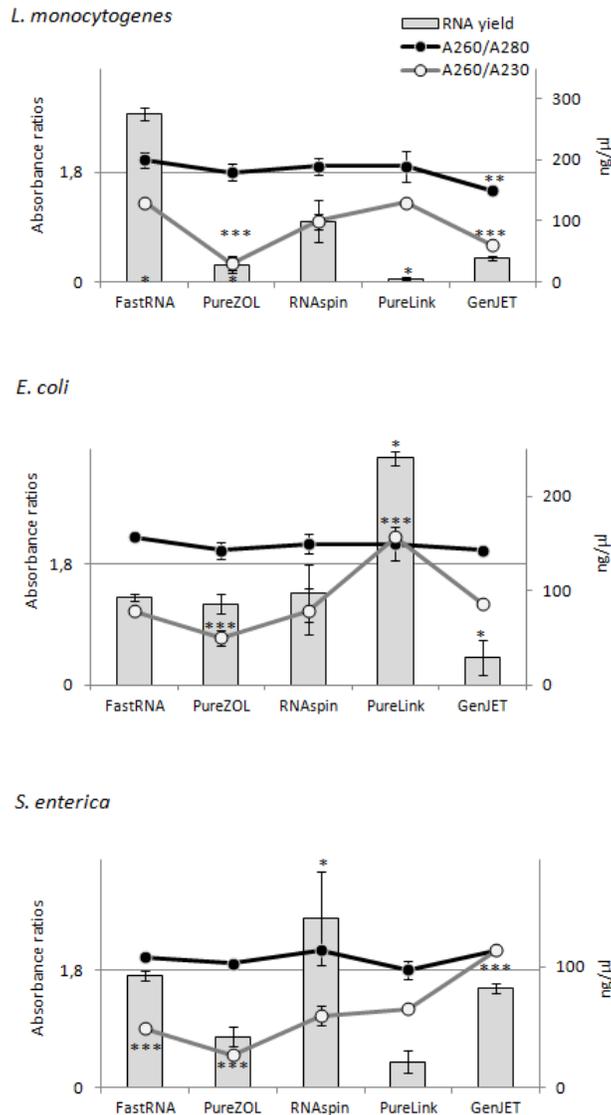


Figure 2 | Comparison of the RNA yield and purity following different RNA extraction procedures. The bars and the points represent the mean plus or minus standard deviation of two independent experiments (*RNA yield, ** A_{260}/A_{280} or *** A_{260}/A_{230} statistically significant $P < 0.05$).

The issue of RNA integrity was assessed by the visualization of the 23S and 16S pattern bands on an agarose gel, as determined by the absence of RNA smearing and by the double intensity of the 23S regarding 16S band [15]. Although this is the most common method used to assess the integrity of total RNA, it has some drawbacks, namely the sensitivity of the assay. On a technical note released by Invitrogen (*Is Your RNA Intact? Methods to Check RNA Integrity*) it was stated that the limit of detection of RNA in an etidium bromide stained agarose gel was 200 ng. However, this limit needs to be determined experimentally, since the quality of the detector and etidium bromide will affect the outcome. With our equipment and experimental setup, we could only detect RNA with at least 1 μg of RNA. Therefore, RNA integrity was not determined in *L. monocytogenes* RNA obtained by PureLink™. With the exception of PureZOL™, all other kits extracted RNA with good integrity (data not shown)

RNA functionality and gene expression

Many other research groups have shown similar studies when addressing the optimization of RNA extraction in different biological samples [10;14;19;20]. However, in such studies, normally they only report the resulted RNA physical characteristics such as the concentration, purity and integrity after different extraction methods. Of utmost importance is to compare the outcome of such physical characteristics in the gene expression quantification. Therefore, to further address the issue of RNA quality and to determine if the RNA extracted was functional, total RNA was reverse transcribed into cDNA and quantified by qPCR. On an extensive study regarding the optimization of eukaryotic cDNA synthesis using commercially available kits, it was found that, when using cDNA synthesized with 40 to 5000 ng of total RNA, there was no significant gene expression variation. However, when using lower concentrations of total RNA, a high variability of gene expression was found [22]. This can be partially explained by the fact that RNA extracted is composed by 1-5 % of mRNA, being the rest rRNA and tRNA. Since mRNA is highly unstable, with half-life in the range of a few minutes, when total RNA yield is very low, some of the specific mRNA can be lost. As a lot less mRNA exists, a small lost can significantly change the outcome of a genetic expression analysis particularly, when small differences in gene expression are being studied. Another possible explanation for this results can be the high sensitivity of qPCR to small amounts of inhibitors in the RNA sample [12].

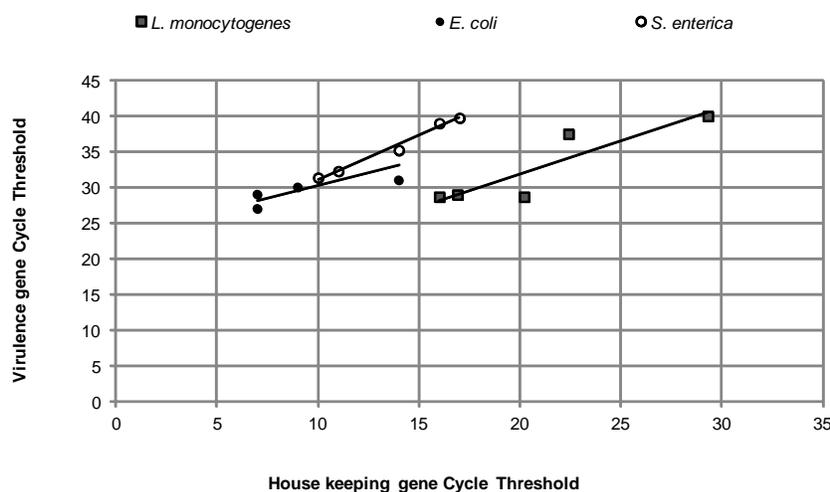


Figure 3 | Gene expression determined from RNA extracted with the different kits. Each point is the average of 2 independent experiments. The standard deviations are less than 1 Ct.

In order to analyze if the RNA's extracted by different kits would impact the gene expression quantification, 7.5 μ L of total RNA, resulting in RNA quantity between the range proposed by Sieber *et al.* [22], was reverse transcribed into cDNA and then quantified by qPCR, using the primers listed in Table 1. The overall results demonstrated that all the kits tested produced functional mRNA, as both housekeeping and virulence genes were detected, despite the differences observed in quantity and purity (Fig. 2). Accordingly to Sieber *et al.* [22], it could be expected that the relationship between housekeeping and virulence gene (known as normalized gene expression) within the same biofilm/bacteria would be constant, since each bacterial biofilm was grown in the same conditions. While for *S. enterica* this was true ($r^2=0.97$), the same did not occur in the other two bacterial biofilms (*E. coli* $r^2=0.65$ and *L. monocytogenes* $r^2=0.63$) (Fig. 3). Analyzing carefully the *E. coli* curve, if we exclude the data obtained by the RNA extracted with PureZOL™, the linearity would be higher ($r^2=0.97$). Since PureZOL™ was extracted with sufficient yield (Fig. 2) to be detected by the agarose gel, we were able to verify that the extracted RNA was not stable confirming, therefore, the crucial role of the RNA integrity in mRNA quantification. The same result was observed with *L. monocytogenes* and this can explain the lower detection of the virulence genes. Nevertheless, in the case of *L. monocytogenes* RNA extracted with PureLink™, we cannot conclude that the variation observed was due to lack of RNA integrity, since the concentration of RNA obtained was lower than the limit of detection.

Conclusions

From our results we can infer that while the RNA extracted by the different kits was functional as cDNA source, since each gene was detected in the qPCR experiment, the outcome of the gene expression quantification was affected by the lower RNA quality. Interestingly, most kits yielded RNA with no protein contamination but with some level of polysaccharides, phenol or chaotropics salts contamination. However, no relationship was found regarding the RNA purity and the variability in gene expression. The same was true for the RNA yield. On the other hand, RNA integrity seemed to be the only factor that impacts directly in the stability of gene expression. Recently, it has been proposed that RNA purity and yield quality indicators were neither sufficient nor straightforward as to determine stability of gene expression [12]. Therefore, it seems clear that a wrong RNA extraction kit selection can have an important impact on genetic expression quantification in biofilms. Moreover, the choice for an RNA extraction kit will most definitively be related to the bacterial species and the composition of the biofilm matrix [7] used since these characteristics impact in the quality of the RNA extracted and thus, in an accurate gene expression quantification.

Acknowledgments

AF and JCB acknowledge the financial support of individual grants SFRH/BD/62359/2009 and SFRH/BD/66250/2009 respectively. The authors acknowledge the gift of bacterial strains to Joana Azeredo and Maria Olivia Pereira.

Conflict of interests

The author(s) declare that they have no conflict of interests.

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