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Article

A Comparison of Plating and Reverse Transcriptase Polymerase Chain Reaction Followed by Microchip Electrophoresis for the Inactivation of *Alicyclobacillus acidoterrestris* Using Saponin

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Foi utilizada a reação em cadeia da polimerase via transcriptase reversa (RT-PCR) seguida por eletroforese capilar em microchip para avaliar a viabilidade de esporos de *Alicyclobacillus acidoterrestris* após a inativação com saponina e temperatura. O método demonstrou ser adequado para tal propósito, além de ser mais rápido e sensível que a tradicional técnica de plaqueamento usado como padrão na indústria de alimentos. Os limites de quantificação e detecção foram 0,0107 e 0,0039 ng μL^{-1} de DNA amplificado, respectivamente. O coeficiente de correlação *(r)* entre plaqueamento e o método desenvolvido foi 0,9977, o qual indica uma excelente correspondência entre eles. Assim, é possível avaliar com confiança a viabilidade da bactéria usando a reação de RT-PCR para detecção. Para avaliação do potencial deste método molecular sobre microbiologia tradicional na inativação de *A. acidoterrestris* foi utilizado saponina como agente de inativação, potencializado com temperatura.

It was used reverse-transcriptase polymerase chain reaction (RT-PCR) followed by capillary electrophoresis on a microchip to probe the viability of *Alicyclobacillus acidoterrestris* spores after inactivation with saponin and heat. The method was shown to be suitable for the purpose, and was faster and more sensitive than the traditional plating technique, the standard of the food industry. The limits of quantification and detection were 0.0107 and 0.0039 ng μL^{-1} of amplified DNA, respectively. The correlation coefficient *(r)* between traditional plating and our method was 0.9977, which indicates an excellent correspondence between them. Consequently, it was possible to assess, with confidence, the viability of bacteria using the RT-PCR reaction for detection. It was evaluated the potential of this molecular method over traditional microbiology in the inactivation of *A. acidoterrestris* by saponin as an effective agent, potentiated by heat.

Keywords: *Alicyclobacillus acidoterrestris,* inactivation, plating, RT-PCR, microchip

Introduction

The physical and chemical characteristics of concentrated orange juice combined with the effects of industrial thermal treatments usually inhibit most pathogens in this beverage.[1] However, the spoilage of fruit juices by the thermoacidophilic spore-forming bacterium *Alicyclobacillus acidoterrestris* has been observed on a worldwide level, with increasing beverage spoilage seen during the last two decades.[2,3] Fresh, concentrated, or even pasteurized orange juices are susceptible to spoilage by spores of *A. acidoterrestris*, which can survive the pasteurization process.[4,5] The bacterium produces guaiacol, which produces flavor taints (off-flavors), and consequently causes the spoilage of fruit juices. However, no toxins are produced by this bacterium.[6]

For the food industry, endospores represent the most difficult life form to inactivate since they show a great resistance not only to heat inactivation, but also to chemical treatments.[7,8] Many compounds have been tested against *A. acidoterrestris* spores, with bacteriocins as the most studied preservatives. Nisin is more effective on spores than on cells; it seems to act at the stage of pre-germinant swelling, and at low concentrations (50-100 IU mL^{-1}), its effect is sporostatic rather than sporicidal.[9,10] Other bacteriocins have been proposed, such as enterocin AS-48, extracted from

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Enterococcus faecalis A-48-32,[11] and warnericin, purified from *Staphylococcus warneri*.[12] Their use, however, is limited due to the cost of extraction and purification.

Another natural compound used to control *A. acidoterrestris* is low molecular weight chitosan (LMW, Mr 50,000-90,000 and 75-85% deacetylation degree). The optimal amount of chitosan added to the growth medium, combined with thermal processing, is 1.4 g L^{-1}; lower amounts are not effective in controlling spore germination.[13] Otherwise, higher amounts result in the formation of flakes, which imparts a negative organoleptic value to the beverage.

The use of active compounds of essential oils has also been studied to control the germination of *A. acidoterrestris*. Cinnamaldehyde, eugenol, and limonene (0.05-0.5 g L^{-1}) were found to inhibit the spores of two different strains of *A. acidoterrestris* (c8 and γ4).[14] Cinnamaldehyde was the most effective compound and a concentration of 0.5 g L^{-1} inhibited spore germination after 13 days. It is possible to point out a hierarchy of the effectiveness of the active essential oil components: cinnamaldehyde > eugenol > limonene. At low concentrations, these chemicals seem to exert a reversible stress, as their effect is time-dependent, i.e., during the outgrowth step.

Saponins are a large group of glycosides, widely distributed in higher plants. The amphiphilic behavior of saponins and their ability to form complexes with steroids, proteins, and membrane phospholipids impart a number of different biological properties. Consequently, these substances present a variety of biological activities, such as molluscicidal, piscicidal, antifungal, antimicrobial, anti-parasitic, anti-inflammatory, analgesic, expectorant, antioxidant, spermicide, and cholesterol-lowering activities.[15-18]

The detection of *A. acidoterrestris* in juices is usually carried out by conventional culture microbiology methods. These methods, however, require several days (5-8 days) to obtain the final result. Additionally, they rely only on presumptive positive or negative results. Thus, molecular methods have the advantage of decreasing the time and increasing specificity and sensitivity in the detection of microorganisms. The polymerase chain reaction (PCR) is the main molecular method used in food analysis, but it cannot distinguish among viable, viable but non-cultivable (VBNC), and dead cells. Therefore, the RNA molecule has been used as the target and amplification template in reverse-transcriptase polymerase chain reaction (RT-PCR) assays. Being a short-lived molecule, RNA works as a marker of viability.[19,20] The use of rRNA to assess viability is supported by the observation that bacterium degradation *in vivo* and *in vitro* is accompanied by ribosome disappearance, which is one of the first ultra-structural signs associated with the loss of bacterial viability.[21]

Traditionally, the use of diagnostics at the molecular level involves a visual inspection of the separation results in an agarose or polyacrylamide gel. However, the need for faster and more sensitive methods jointly with technological advances has enabled the development of new methodologies. With progress in microfluidics, new systems for capillary electrophoresis-based lab-on-a-chip have been developed; these techniques represent a good alternative to rapid and sensitive gel electrophoresis. The Agilent 2100 Bioanalyzer was the first commercially available system to utilize chip-based nucleic acid separation technology. Using this instrument, nucleic acid samples are automatically separated and analyzed by capillary electrophoresis (CE) with laser induced fluorescence (LIF) detection.[22,23] Additionally, the microfluidic system directly provides quantification data with excellent sensitivity.

The purpose of this work was to correlate the plating technique (used as the reference method)[24] and the new RT-PCR method coupled with microchip detection in the inhibition of *A. acidoterrestris* spores in orange juice by saponin and thermal treatments.

Experimental

Cell and culture media

Alicyclobacillus acidoterrestris CCT 49028 were provided from the André Tosello Tropical Culture Collection (Campinas, São Paulo, Brazil). This extracted strain was selected to study the inactivation because orange juices are contaminated by it. Strains were grown at 45 °C for five days in yeast glucose starch (YGS) media composed of 2.0 g of yeast extract, 1.0 g of glucose, 2.0 g of starch in 500 mL distilled water. The pH was adjusted to 3.7 with 1.0 mol L^{-1} H_2SO_4.

The spores, obtained from cultures grown at 45 °C for 5 days, were spread onto YGS agar in Petri dishes and incubated at 45 °C for 5 days. After reaching more than 90% sporulation, spores were collected with a sterile swab and suspended in sterile distilled water. The pool of spores collected from the different plates was centrifuged at 5000 × g for 15 min at 4 °C, washed twice with sterile distilled water by repeated centrifugation, and finally suspended in acidified sterile distilled water (pH 3.7). The spores were stored in tubes at −20 °C until use. The suspensions with spores were activated by heat shock at 80 °C for 10 min before inoculation into orange juice.

Juices

In this work, it was used concentrated orange juice (66.5 ºBrix, pH 3.57), commercially available in local

stores, as a matrix for experiments on *A. acidoterrestris* inhibition.

RNA total extraction

All glassware used for RNA extraction was baked at 180 °C overnight and all reagents were treated with 1% diethylpyrocarbonate (DEPC). Total RNA was extracted from 1.0 mL of samples using Trizol reagent (Invitrogen - Carlsbad, CA, USA). The pellets were suspended in H_2O-DEPC treated with DNase at 30 °C for 20 min to eliminate DNA molecules.

Reverse transcription and PCR reactions

For RT-PCR reactions, specific primers were designed for regions identified in previous study from the sequencing of 16S rRNA.[25] The pair of primers yielded a PCR product of 294 bp (base pairs). The forward and reverse primer sequences were:

forward (Ba190F):

5'-AC(A/G)GGTAGGCATCTCTTGT-3' and

reverse (Ba490R):

5'-AGGAGCTTTCCACTCTCCTTGT-3'

The reverse-transcription step for the generation of cDNA was performed in a final volume of 20 µL. Antisense primer (1 µmol L^{-1}) and 1 µL RNA samples digested with DNase I were denatured for 5 min at 70 °C, then immediately cooled on ice for 5 min and added to 15 µL RT mix (2.0 mmol L^{-1} dNTPs, 3 mmol L^{-1} $MgCl_2$, 1× RT buffer and 1 µL ImProm-II™ RT (Promega, Madison, WI, USA). The mixture was incubated for 5 min at 25 °C, 60 min at 40 °C, and 15 min at 70 °C. After synthesis of the complementary cDNA strand, PCR was performed in 13 µL total reaction volume containing 3 µL cDNA product, 1× PCR buffer, 1.5 mmol L^{-1} $MgCl_2$, 0.2 mmol L^{-1} dNTPs, 1 U *Taq*-DNA polymerase (Invitrogen), and 1 µmol L^{-1} of the specific primers. The PCR was performed on a conventional thermocycler (Mastercycler Gradient, Eppendorf, Germany) and cycle conditions were as follows: initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 54 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 7 min. All experiments were performed in triplicate for each amplification reaction.

Evaluation of the analytical parameters of the Agilent 2100 Bioanalyzer

These experiments aimed to demonstrate the quality and reliability of analytical measurements performed using the microchip CE system for this work, in which some important analytical figures of merit were evaluated, such as linearity and dynamic range, accuracy, and limits of detection and quantification. The linearity and dynamic range of the Agilent 2100 Bioanalyzer were evaluated by constructing an analytical calibration curve using different concentrations of a 294 bp amplicon obtained as described in the Reverse transcription and PCR reactions section. The analytical curve was established with DNA concentrations ranging from 0.01 to 50 ng μL^{-1}. To evaluate the accuracy of the proposed method, DNA was diluted into six different concentrations (50.0, 25.0, 12.5, 6.25, 3.12, and 1.56 ng μL^{-1}) within the linear range. Finally, it was used the following equations to evaluate the detection (LOD) and quantification limits (LOQ):

$$LOD = 3.3 \times \frac{SD}{S} \quad (1)$$

$$LOQ = 10 \times \frac{SD}{S} \quad (2)$$

where SD is the standard deviation and S is the slope of the curve.

Detection of amplification products by microchip electrophoresis

Separation, detection, and quantification of the RT-PCR amplification were accomplished using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbron, Germany) with the LabChip® DNA 7500 kit. Samples of nucleic acids were automatically separated and analyzed by microchip capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection by monitoring the emission between 670 nm and 700 nm. Each chip analyzes 12 samples simultaneously in approximately 30 min, using only 1 µL of sample.

Comparison of *A. acidoterrestris* detection by plating and RT-PCR

The comparison of *A. acidoterrestris* quantification by plating techniques and by RT-PCR was carried out by diluting an volume with a known count of *A. acidoterrestris* spores, then inoculating it into concentrated orange juice. The initial concentration was 1×10^4 CFU mL^{-1} and it was monitored by optical density at 600 nm. Starting from the initial concentration, serial dilutions were made until 0.1 CFU mL^{-1} was produced. Again, the number of colonies was counted by plating and RT-PCR. Each sample, with different CFU counts, yielded different concentrations of DNA, and were run on the Bioanalyzer 2100 microchip in triplicate. Additionally, each assay was

made in quintuplicate for each dilution in Petri plates, and the experiments for all concentrations were repeated three times on different days.

Effect of heat-treatment and saponin on *Alicyclobacillus acidoterrestris* spores in concentrated orange juice

In this experiment, the inactivation of *A. acidoterrestris* was performed with a commercial source of saponin and a purified extract of saponin from the fruit of *Sapindus saponaria,* in concentrations ranging from 100 to 500 mg L^{-1}.

Concentrated orange juice was inoculated with 1×10^4 CFU mL^{-1} spores of *A. acidoterrestris* and then either a commercial saponin solution or a purified extract diluted in water was added to the inoculated juice at a given concentration directly in test tubes. Other juice samples inoculated with spores were heated at 99 °C for 1 min. This treatment was chosen because this was the best thermal treatment condition obtained in previous studies.[26] CE on the microchip system (Agilent 2100 Bioanalyzer) quantified the DNA in triplicate, and the plating assays were done in quintuplicates.

Results and Discussion

Evaluation of the analytical parameters of the Agilent 2100 Bioanalyzer

Linearity and dynamic range

Figure 1 shows the linear behavior for the entire range with a correlation coefficient $r = 0.9995$. The r parameter allows us to estimate the quality of the data, indicating the dispersion of the experimental points and the uncertainty of the regression coefficients. High sensitivity and low detection limits are the differential advantage of this equipment. Moreover, using traditional DNA quantification methods would not achieve the desired accuracy.

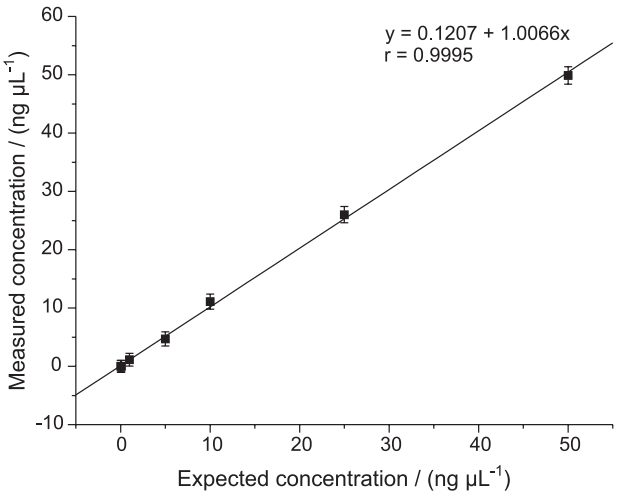

Figure 1. Analytical calibration for the estimation of linear range, limits of detection and quantification, and accuracy of Agilent 2100 Bioanalyzer.

Accuracy

It is very important to adopt sensitive techniques with high accuracy in the evaluation of cell viability. As said before, the accuracy of the method was evaluated into six different concentrations (50.0, 25.0, 12.5, 6.25, 3.12, and 1.56 ng μL^{-1}) within the linear range. Table 1 shows the average of measurements of concentration of DNA performed on the Agilent 2100 Bioanalyzer, the relative standard deviation (RSD), and the difference from the expected value. It could be observe that the values obtained for concentration did not exceed 2.5% of the expected value. This low deviation value indicates that the equipment provides a high accuracy in the quantification of a transcript DNA. It was noticed that the RSDs were higher for analyses performed on different days. While this value did not exceed 6.0% in intra-day determinations, it reached 9.4% in inter-day experiments. Such values, however, are still acceptable for most analytical guidelines and indicate that the equipment shows good reproducibility. The data obtained here are in agreement with those from other authors,[27-29] who evaluated the convenience of the use of the Agilent 2100 Bioanalyzer for quantification studies and routine analysis of DNA.

Table 1. Precision and accuracy of the quantification of the DNA amplicon

Expected concentration / (ng μL^{-1})	Intra-assay			Inter-assay		
	Average / (ng μL^{-1})	RSD / %	Difference / %	Average / (ng μL^{-1})	RSD / %	Difference / %
50.0	49.8	2.28	–0.4	49.2	3.06	–1.6
25.0	25.2	3.02	0.8	25.3	4.39	1.2
12.5	12.3	4.02	–1.6	12.2	6.36	–2.4
6.25	6.29	3.89	0.6	6.40	4.52	2.4
3.12	3.16	5.79	1.3	3.18	9.35	1.9
1.56	1.57	5.97	0.6	1.59	8.18	1.9

Limits of detection and quantification

The limit of detection and the limit of quantification are the minimum concentrations of analyte that can be reliably detected or accurately measured, respectively.

Table 2 shows the values derived from the analytical curves of two different assays (to increase the stringency of the study) and the calculated limits of detection and quantification, which were 0.0039 and 0.0107 ng μL^{-1}, respectively. The values found are quite low, and such values cannot be reliably obtained with other methods using biological and microbiological processes. Thus, the results show that the proposed method is highly sensitive for detecting and quantifying DNA samples from bacterial transcripts with high bioanalytical standards. The results of the analytical parameters from the Agilent 2100 Bioanalyzer presented here showed the potential of capillary electrophoresis on a microchip platform for the analysis of cellular viability with the advantage of i) high speed, ii) high accuracy, iii) good reliability, and iv) high sensitivity, yet with low sample consumption.

Table 2. Limits of detection and quantification for a DNA transcript performed in the Agilent 2100 Bioanalyzer

	LOD	LOQ
Average of y-intercepts from the calibration curves	2.11×10^{-4}	-1.13×10^{-4}
Standard deviation of the y-intercepts (SD)	0.0012	0.0012
Average slope of the calibration curves (S)	1.0071	1.0038
Calculated value of the limit / (ng μL^{-1})	0.0039	0.0107
Corresponding value in CFU mL^{-1}	0.8725	0.8755

Comparison of detection of *A. acidoterrestris* by plating and molecular techniques

Plating is the microbiology method commonly applied for the detection of viable bacteria. This technique takes the advantage of detecting only living cells capable of replication. Since plating is the industry standard, this technique was used as a comparison parameter for other, newer detection methods, i.e., RT-PCR.

We propose using a microfluidic platform in conjunction with molecular methods as an alternative to plating techniques for the fast detection of viable organisms. In contrast to the qualitative nature of plating, we also propose imposing a quantitative aspect with strong analytical criteria. First, it was directly compared the correlation between the CFU counts in a serial dilution of *A. acidoterrestris* with the concentration of the amplicon generated by RT-PCR. The direct correlation between the two is shown in Figure 2.

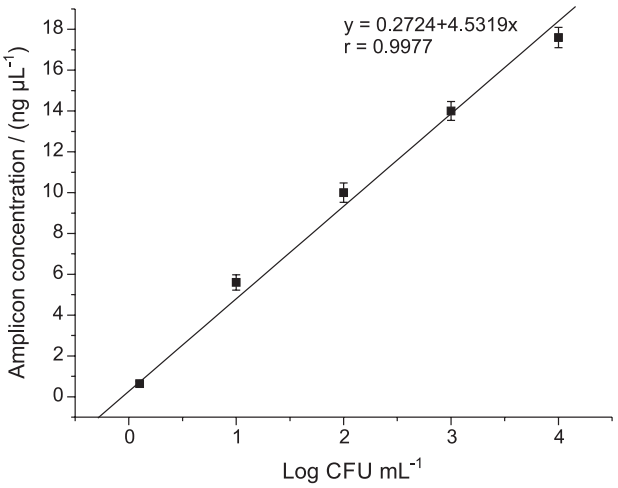

Figure 2. Comparison of detection by plating and RT-PCR of *Alicyclobacillus acidoterrestris* spores in concentrated orange juice.

The correlation coefficient (r) was 0.9977, which indicates an excellent correspondence between the evaluated methods, while the determination coefficient (r^2) of 0.9954 indicates that more than 99.5% of the data are explained by the variance of the data. Consequently, it is possible to assess with high analytical confidence that the viability of bacteria as spores in terms of CFU mL^{-1} is related to the concentration of the amplicon detected by RT-PCR. The initial inoculum of 1×10^4 CFU mL^{-1} was diluted to 1×10^{-1} CFU mL^{-1}, which was the lowest dilution detectable in the plating method under the parameters defined in this work. Yet, the high sensitivity of microchip CE with LIF detection by the Agilent 2100 Bioanalyzer coupled with the amplification power of the PCR reaction and the specificity of the reverse-transcriptase reaction towards viable organisms allowed dilutions as low as 0.8725 CFU mL^{-1} for detection and 0.8755 CFU mL^{-1} for quantification of viable cells. Thus, it is unquestionable that the method for viability detection of microorganisms by RT-PCR is more sensitive and faster than the traditional plating method used in applied microbiology.

Application of CE microchip with RT-PCR to investigate the inactivation of *A. acidoterrestris* in orange juice

It was explored the high sensitivity capability of the RT-PCR/Bioanalyzer method in a study of the inactivation of bacteria. The use of saponin combined with heat (99 °C for 1 min) aimed to inactivate the growth of *A. acidoterrestris*. Heat promotes a substantial decrease in the initial number of viable bacteria, while the presence of saponin inhibits the remaining microorganisms.[26]

Figure 3 shows the growth behavior of the bacteria over time after treatment. A reduction of 96.4% for both

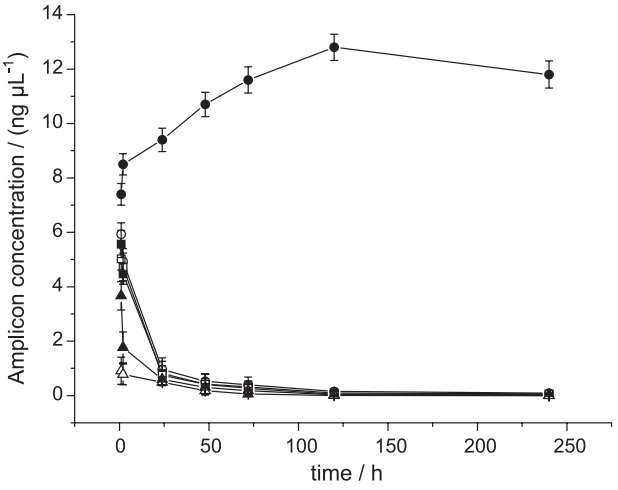

Figure 3. Curves for viability of spores in concentrated juice treated at 99 ºC for 1 min in presence of saponin at concentration of (●) zero (control), (○) 100 mg L^{-1}, (■) 200 mg L^{-1}, (□) 300 mg L^{-1}, (▲) 400 mg L^{-1} and (△) 500 mg L^{-1} of saponin in the growth medium.

methods used was achieved after 240 h (10 days) with the addition of 100 mg L^{-1} commercial saponin. Without the initial thermal treatment, inactivation was only 76.0%, thus the thermal treatment applied before saponin addition potentiated bacterial inactivation by 20.4%. If a higher concentration of saponin was used, over 99.0% inactivation was achieved within 120 h (5 days) at all concentrations of the preservative. The results demonstrate that thermal treatment was able to potentiate the inactivation the *A. acidoterrestris* by saponin. Heat reduces the initial number of cells, and a given concentration of saponin is able to suppress the remaining cells.

For this inactivation process to be used in the fruit juice industry, a concentration of saponin starting from 300 mg L^{-1} would be recommended. At this concentration, a 98.8% reduction is possible after 120 h in concentrated juice. This kind of juice is often exported overseas to other countries and the time for transportation is long. Thus, inhibition could be achieved during the journey. Thereby, when reconstituted for consumption, the juice will not present contamination problems with *A. acidoterrestris* and not compromise the organoleptic properties of the juice, typically diluted ten-fold.

It is possible to see in Table 3 that the plating method was not able to detect all surviving spores at higher concentrations of saponin or longer incubation times because it is not as sensitive as the method proposed in this work. Thus, the RT-PCR method followed by electrophoresis in a microchip is more suitable for the detection of microbial viability than the conventional plating assay.

Conclusions

It was applied in this work a method to detect and quantify *A. acidoterrestris* inactivation in juice. By applying RT-PCR to *A. acidoterrestris* treated with saponin and heat, we were able to show that a lab-on-a-chip system, i.e., the Agilent 2100 Bioanalyzer, can be used for the rapid, sensitive, and semiquantitative detection of cell viability. We demonstrated a linear correlation between traditional plating and our novel method with a correlation

Table 3. Results on the viability of *A. acidoterrestris* spores in concentrated orange juice after treatment with saponin at different concentrations (mg L^{-1}) and heating at 99 °C for 1 min. Comparison of the plating technique and RT-PCR by CE microchip

time / h	Log_{10} CFU mL^{-1}				
	100	200	300	400	500
0	3.80 ± 0.19	3.80 ± 0.25	3.84 ± 0.19	3.80 ± 0.25	3.84 ± 0.20
24	1.66 ± 0.24	1.51 ± 0.22	1.26 ± 024	0.98 ± 0.22	0.82 ± 0.25
48	1.59± 0.19	1.35 ± 0.20	1.21 ± 0.19	0.98 ± 0.20	0.75 ± 0.26
72	1.33 ± 0.23	1.09 ± 0.21	1.08 ± 0.23	0.83 ± 0.21	0.51 ± 0.16
120	0.46 ± 0.20	0.13 ± 0.27	0.00 ± 0.03	0.00 ± 0.02	0.00 ± 0.04
240	0.12 ± 0.24	ND	ND	ND	ND
time / h	Amplicon concentration / (ng μL^{-1} ± SD)				
	100	200	300	400	500
0	17.6 ± 0.43	17.6 ± 0.30	17.8 ± 0.43	17.6 ± 0.38	17.9 ± 0.30
24	7.85 ± 0.53	7.15 ± 0.35	6.01 ± 0.29	4.74 ± 0.45	4.01 ± 0.43
48	7.55 ± 0.49	6.45 ± 0.39	5.81 ± 0.34	4.75 ± 0.38	3.71 ± 0.39
72	6.35 ± 0.39	5.25 ± 0.27	5.21 ± 0.40	4.05 ± 0.36	2.61 ± 0.41
120	2.35 ± 0.36	0.85 ± 0.10	0.21 ± 0.07	0.15 ± 0.05	0.09 ± 0.02
240	0.80 ± 0.10	0.40 ± 0.10	0.06 ± 0.01	0.04 ± 0.01	0.03 ± 0.01

ND: not detected.

coefficient (r) of 0.9977. However, due to the high sensitivity of microchip CE with LIF detection using the Agilent 2100 Bioanalyzer coupled with the amplification power of the PCR reaction and the specificity of the reverse-transcriptase reaction, this new technique allowed dilutions as low as 0.8725 CFU mL^{-1} for detection and 0.8755 CFU mL^{-1} for quantification of viable cells. Thus, the method for microbial viability detection developed in this work is more sensitive and faster than the traditional plating method used in applied microbiology.

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References

1. Eguchi, S. Y.; Manfio, G. P.; Pinhatti, M. E. M. C.; Azuma, E.; Variane, S. F.; *Acidothermophilic Sporeforming Bacteria (ATSB) in Orange Juices: Detection Methods, Ecology and Involvement in the Deterioration of Fruit Juices*, 2nd ed.; Fundação André Tosello & ABECitrus: Campinas, 1999, vol. 1.
2. Eiroa, M.; Junqueira, V.; Schmidt, F.; *J. Food Protect.* **1999**, *62*, 883.
3. Pettipher, G. L.; Osmundson, M. E.; Murphy, J. M.; *Lett. Appl. Microbiol.* **1997**, *24*, 185.
4. Splittstoesser, D. F.; Churey, J.; Lee, C. Y.; *J. Food Protect.* **1994**, *57*, 1080.
5. Yamazaki, K.; Tekuda, H.; Shinano, H.; *Biosci. Biotechnol. Biochem.* **1996,** *60*, 543.
6. Orr, R. V.; Shewefelt, R.; Huang, C.; Tefera, S.; Beuchat, R. L.; *J. Food Protect.* **2000**, *63*, 1517.
7. Knorr, D.; *New Methods of Food Preservation*, 2nd ed.; Blackie: London, 1995, vol. 1.
8. Butz, P.; *Food Res. Internat.* **2002**, *35*, 279.
9. Komitopoulou, E.; Boziaris, I. S.; Davies, E. A.; Delves-Broughton, J.; Radstrom, M. P.; *Int. J. Food Science Technol.* **1999**, *34*, 81.
10. Yamazaki, K.; Murakami, M.; Kawai, Y.; Inoue, N.; Matsuda, T.; *Food Microbiology* **2000**, *17*, 315.
11. Grande, M. J.; Lucas, R.; Abriouel, H.; Omar, N. B.; Maqueda, M.; Martínez-Bueno, M.; Martínez-Cañamero, M.; Valdivia, E.; Gálvez, A.; *Int. J. Food Microbiology* **2005**, *104*, 289.
12. Minamikawa, M.; Kawai, Y.; Inoue, N.; Yamazaki, K.; *Curr. Microbiology* **200**5, *1*, 22.
13. Falcone, P. M.; Campaniello, D.; Altieri, C.; Sinigaglia, M.; Corbo, M. R.; Anese, M.; Del Nobile, M. A.; *Ital. J. Food Sci.* **2005**, *special issue*, 142.
14. Bevilacqua, A.; Corbo, M. R.; Sinigaglia, M.; *Int. J. Food Sci. Technol.* **2008**, *43*, 1271.
15. Hostettmann, K.; Marston, A.; *Chemistry and Pharmacology of Natural Products*, 3rd ed.; Cambridge University Press: Cambridge, 1995, vol. 1.
16. Francis, G.; Kerem, Z.; Makkar, H. P. S.; Becker, K.; *Brit. J. Nutrit.* **2002**, *88*, 587.
17. Sparg, S. G.; Light, M. E.; Van Staden, J.; *J. Ethnopharmacol.* **2004**, *94*, 219.
18. Tsuzuki, J. K.; Svidzinski, T. I. E.; Shinobu, C. S.; Silva, L. F. A.; Rodrigues-Filho, E.; Cortez, D. A. G.; Ferreira, I. C. P.; *Anais Acad. Bras. Cienc.* **2007**, *79*, 577.
19. Sheridan, G. E.; Masters, C. I.; Shallcross, J. A.; MacKey, B. M.; *Appl. Environ. Microbiol.* **1998**, *64*, 1313.
20. Williams, J. M.; Trope, M.; Caplan, D. J.; Shugars, D. C.; *J. Endod.* **2006**, *32*, 715.
21 McKillip, J. L.; Jaykus, L. A.; Drake, M.; *Appl. Environ. Microbiol.* **1998**, *12*, 4264.
22. Nachamkin, I.; Panaro, N.; Huong Ung, M. L.; Kricka, L.; Wilding, P. J.; *J. Clin. Microbiol.* **2001**, *39*, 754.
23. Gottwald, E.; Müller, O.; Polten, A.; *Electrophoresis* **2001**, *22*, 4016.
24. Jassona, V.; Jacxsens, L.; Luning, P.; Rajkovic, A.; Uyttendaele, M.; *Food Microbiol.* **2010**, *27*, 710.
25. Yamazaki, K.; Tekuda, H.; Inoue, N.; Shinano, H.; *Letters in Appl. Microbiology* **1996**, *2*, 350.
26. Alberice, J. V.; Funes-Huacca, M. E.; Guterres, S. B.; Carrilho, E.; *Int. J. Food Microbioogy.* **2012**, *159*, 130.
27. Panaro, N. J.; Yuen, P. K.; Sakazume, T.; Fortina, P.; Kricka, L. J.; Wilding, P.; *Clin. Chem.* **2000**, *11*, 1850.
28. Jabasini, M.; Zhang, L.; Dang, F.; Xu, F.; Almofli, M. R.; Ewis, A. A.; Lee, J.; Nakahori, Y.; Baba, Y.; *Electrophoresis* **2002**, *23*,1537.
29. Sodowich, I.; Fadl, I.; Burns, C.; *Electrophoresis* **2007**, *28*, 2368.

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